Original Article

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Methylation Analysis of OCT 4 Gene in AML and ALL Patients Using Sodium Bisulfite

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Conflict of interest: None
Funding source: None

Article received: 09-11-2025
Article accepted: 17-12-2025

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DOI: 10.65433/mjms.v3i2.104.

ABSTRACT

Introduction: Methylation is a key epigenetic mechanism regulating gene expression. The OCT4 gene, essential for stem cell pluripotency, is normally silenced by methylation after embryonic development. Dysregulation of this process is implicated in cancer, including leukemia.

Objective: This study aimed to analyze the methylation pattern of the OCT4 gene in patients with Acute Myeloid Leukemia (AML) and Acute Lymphoid Leukemia (ALL).

Methodology: In a case-control design, 40 samples each from AML patients, ALL patients, and healthy controls were collected from PIMS Islamabad. Diagnosis was confirmed via blood count and bone marrow biopsy. Genomic DNA was extracted, quantified, and treated with bisulfite. PCR was performed using designed primers to assess the methylation status of the OCT4 gene.

Results: Clinical analysis revealed significantly elevated white blood cell counts in leukemia patients compared to controls, with a higher disease prevalence in males. Epigenetic analysis showed that the OCT4 gene was predominantly hypomethylated in both AML and ALL patient samples. Successful PCR amplification after bisulfite treatment confirmed the conversion of unmethylated cytosines, validating the observed hypomethylation.

Conclusion: The study concludes that hypomethylation of the OCT4 gene is a common feature in AML and ALL, suggesting its potential role in disrupting normal cellular differentiation and contributing to leukemogenesis. Further research is needed to fully elucidate the mechanistic role of OCT4 methylation in these cancers.

Keywords: OCT4 gene, Acute Myeloid Leukemia, Acute Lymphocytic Leukemia, Methylation, Epigenetics.

Introduction

Leukemia is a type of cancer that resides in the blood, therefore it is known as blood cancer.[1] Leukemia is divided into 4 types: Acute myeloid leukemia (AML), acute lymphoid leukemia (ALL), chronic myeloid leukemia (CML), and chronic lymphoid leukemia (CLL).[2] A type of leukemia in which disorder occurs in lymphoid predecessor cells. Children and adults are both affected by ALL. Advancement in treatment has led us to achieve an 80% cure rate.[3] In AML, a disorder of hematopoietic stem cells occurs, which halts the process of hematopoiesis, causing the production of a clonal population of blasts or neoplastic cells.[4] With the help of standard chemotherapy, the remission rate of AML is about 50% to 85%.[5]

Previous studies suggest that in patients of AML/ALL, somatic mutations do occur in the gene of *DNMT3A*.[6] These mutations can lead to leukemogenesis and drug resistance. The pattern of DNA methylation changes after the synthesis of blood cells from hematopoietic stem cells.[7] The transfer of information according to gene sequences is termed genetics. The stable, mitotically preserved regulatory mechanism of gene expression, with a conserved coding sequence, is termed epigenetic.[8] Epigenetic changes can cause carcinogenesis by the silencing of some important genes.[9]

DNA methylation is one of the most stable modifications. In mammalian DNA, methylation is restricted to cytosine residues followed by a guanosine residue (the CpG dinucleotide).[10] DNA methyltransferases (DNMTs) are the enzymes responsible for the methylation of these repeats.[11] Three proteins, DNMT1, DNMT3A and DNMT3B, have been observed to have methyltransferase catalytic activity in mammalian cells.[11]

A couple of mechanisms have been recommended for transcriptional repression by means of DNA methylation. It can directly suppress the binding of transcription factors (TFs) or involve

the binding of proteins to m5CpG dinucleotides.[12] The hallmarks of cancer include epigenetic defects such as hypomethylation of the genome and hypermethylation of CpG islands. In hypomethylation, transposable elements and repetitive regions of DNA are highly involved. In hypermethylation, tumor suppressor gene promoters are mostly involved. [13]

Mammalian embryo growth is controlled by some regulatory genes which in turn regulate the expression of different genes.[14] *OCT4* is present on chromosome 6p21.3.[15] It is observed that OCT4 is expressed in all blastomeres at early stages, but then its expression gets limited to the inner cell mass (ICM) and is deregulated in the trophectoderm (TE) and the embryonic endoderm.[16] At later stages, OCT4 expression remains confined to developing germ cells. OCT4A is present in the nucleus and functions as a transcription factor. OCT4B is found in the cytoplasm.[17]

OCT4 must be carefully regulated to ensure proper differentiation of the germline and various tissue organs. In mice, OCT4 mRNA is found in mature oocytes. When the cell reaches the eight-cell stage, OCT4 expression is at a higher level.[18] At later stages, its 0expression is deregulated in the TE and gets limited to the ICM.[19] At the transcription level, OCT4 expression is regulated by *cis*-acting elements located upstream of the *OCT4* gene and by chromatin methylation.[20] Experiments carried out on ES cells indicate that OCT4 controls the pluripotency of stem cells in a quantitative manner; it was reported that higher OCT4 expression drives ES cells to mesoderm and endoderm, while low expression differentiates ES cells into TE.[21] These results point out that OCT4 regulation is different from the normal regulation of genes, as it has a role in both the "on" and "off" states.

There are many methods for the analysis of methylation. It can be non-specific and gene-specific methods. Restriction endonucleases check any DNA in the cell for correct and incorrect methylation patterns and cleave any incorrect methylation pattern. In this way, most foreign DNA will be recognized and degraded by endonucleases before they express.[22] Bisulfite genomic treatment is used for a quantitative, qualitative, and efficient approach to detect 5-methylcytosine at single base pair resolution.[23]

The major objectives of the study were:
- To optimize the Bisulfite treatment protocol and authentication by Bisulfite-specific primers.
- To compare the methylation pattern of the upstream regulatory region of the *OCT4* gene (up to 1500 bp upstream region from the first coding exon) in blood cells of normal, acute myeloid leukemia, and acute lymphoid leukemia patients.
- To sequence the amplified region for in-depth analysis of the methylation pattern.

Methodology

The study was conducted on 120 blood samples 40 each of Control, ALL and AML patients. The blood samples were collected from PIMS hospital Islamabad. TLC count and Bone marrow biopsy was used for the identification of ALL and AML patients. 5ml of blood was collected from AML, ALL patients and also from control individuals in Vacutainer tubes containing ethylene diaminetetraacetate (EDTA) (BD Vacutainer ® k3 EDTA, Franklin Lakes NJ, USA). The samples were stored at 4ºC till DNA extraction.

DNA Extraction:

Phenol choloroform method (24) was used for the extraction of genomic DNA from blood samples.

Agarose Gel Electrophoresis:

1% agarose gel was used to check the extracted DNA. For the preparation of 1% agraose gel, mixture of 100 ml buffer was made, which contains 10ml of 10X TBE (Tris, EDTA, boric acid) buffer and 90ml of distilled water. To this mixture 1g of agarose was added and dissolved by heating at high temperature in microwave oven for 2 minutes. 10 µl of ethidium bromide was added for tracking of DNA.

The solution was poured into a gel casting plate and allowed to solidify at room temperature. 5 µl of DNA was mixed with 5 µl of bromophenolblue (loading dye) and loaded in the wells of gels. In the gel tank 1000ml of 1X TBE (100ml 10 X TBE and 900ml distilled water) buffer was poured to carry out electrophoresis for one and half an hour at 90 volts. under UV transilluminator (Biometra, Gottingen, Germany) the gel was illuminated to visualize the DNA and with the help of digital camera DC 290 (Kodak, New York, USA) gel picture was captured.

Table I. Sequence of Conventional primers			
Primer	Sequence	Product Size	Tm
Oct- F1 **Oct- R2**	5/ AGATAGAGCCACTGACCCCA 3/ 5/ ACATCCCTCAATCTGCCAGG 3/	500bp	62.4°C 62.4°C
Oct- F2 **Oct- R2**	5/ GGAGTTGAAAGTTGGGTGTG 3/ 5/ CAAGCCCTCATTTCACCAGG 3/	551bp	60.4°C 62.4°C
Oct- F3 **Oct- R3**	5/ GGATTGCTTTGGCCCAGTAG 3/ 5/ AGAGGGGTTGAGTAGTCCCTT 3/	350bp	58°C 59°C

Table II. Sequence of Bisulfite specific primers			
Primer	Sequence	Product size	Tm
BSP-F1 **BSP-R1**	5/ TTTATTTAGTTATGTTAGTTGTTTTAT3/ 5/ GGTTTTTTGTTTAGGTTAGAAATATAT3/	493bp	53°C 56.5°C
BSP-F2 **BSP-R2**	5/ GGTTTTTTGTTTAGGTTAGAAATATAT3/ 5/ GAAGGTAGATAGAGTTATTGATTTTAGT3/	700bp	55.5°C 58°C

Genomic DNA Quantification: By taking the optical density (OD) at 260 nm Genomic DNA was quantified in Generay UV- photometer (Biometra, Goettingen, Germany) and for polymerase chain reaction it was

subsequently diluted to 40-50 ng/ul

Bisulfite Treatment: There are two methods for bisulfite treatment:

1.Commercially available kits

2.Conventional methods

We have used conventional method for bisulfite genomic treatment.

Polymerase Chain Reaction: The PCR amplified products were analyzed on 2% agarose gel. Primers were designed for the 1500bp 5/ upstream regulatory region from the first coding exon of *OCT4* gene. 3 set of primers were designed.

Results

Analysis of Clinical parameters for Acute Myeloid leukemia: Mean value of AML patients were significantly high as compared to the control group. High standard deviation value of AML patients shows that it is more scattered from mean because of the elevated WBC count. T test confirmed that im AML patients WBC count is higher than in control group. P value from chi square test was statistically significant showing prominent difference between two variables i.e control and AML group. Table shows data of Mean, SD,SE, P value and Chi square test of control and AML group. Figure shows graph plotted between WBC count of control and ALL group.

Table III. Statistical Parameters of WBC Count of control and AML samples		
	Control WBC count	AML WBC Count
Mean	7272.475	13284.83
Standard Dev.	2527.26	7276.366
Standard Error	398.6215	1147.692
P value (T test)	< 0.0001	
Chi square (χ^2)	93310,39 (<0.0001)	

Analysis of Clinical parameters for Acute Lymphocytic leukemia: Mean value of ALL patients were significantly high as compared to the control group. High standard deviation value of ALL patients shows that it is more scattered from mean

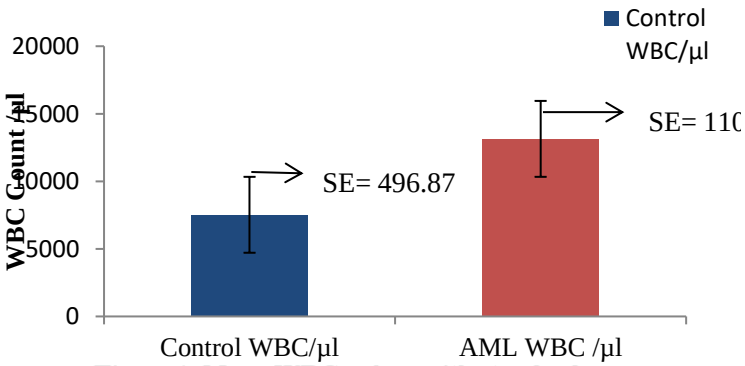

Figure 1. Mean WBC values with standard error

because of the elevated WBC count. T test confirmed that in ALL patients WBC count is higher than in control group. P value from chi square test

was statistically significant showing prominent difference between two variables i.e control and ALL group. Table shows data of Mean, SD,SE, P value and Chi square test of control and ALL group. Figure shows graph plotted between WBC count of control and ALL group.

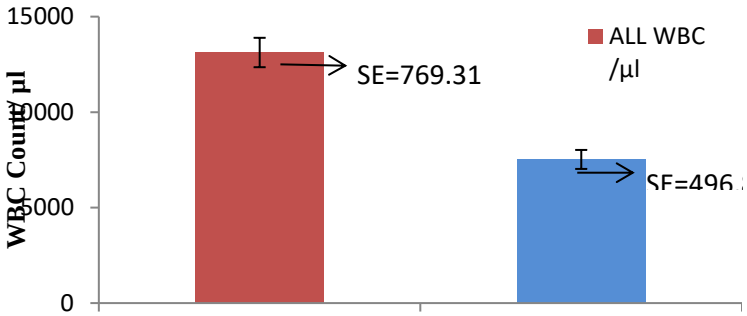

Figure 2. Mean WBC values with standard error

Amplification of *OCT4* gene in Normal, AML and ALL patients

With conventional primers:

Amplification of *OCT4* gene was done in control, AML and ALL patients with conventional primers. The results of amplification are shown in fig.10, 11 and 12.All the samples show bands with conventional primers None of the samples were bisulphite treated.

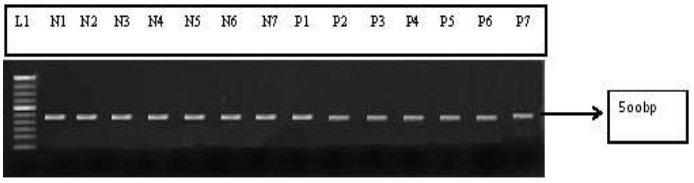

Figure 3: Electropherogram of 2% agarose gel showing amplified products of 500 bp by control primer OCT-F1 and OCT-R1.

The lane L1 represents 100 bp ladder while lanes P1 to P4 represents AML, lanes N1 to N7 represents control group, lanes P5 to P7 represents ALL lanes.

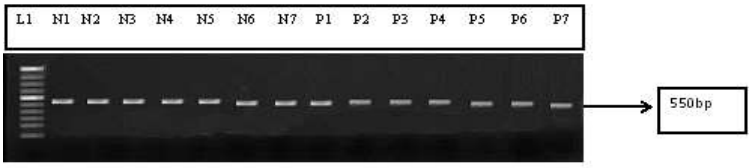

Figure 4: Electropherogram of 2% agarose gel showing amplified products of 550 bp by control primer OCT-F2 and OCT-R2.

The lane L1 represents 100 bp ladder while lanes N1 to N7 represents control group, lanes P1 to P3 represents AML patients, lanes P4 to P7 represents ALL lanes

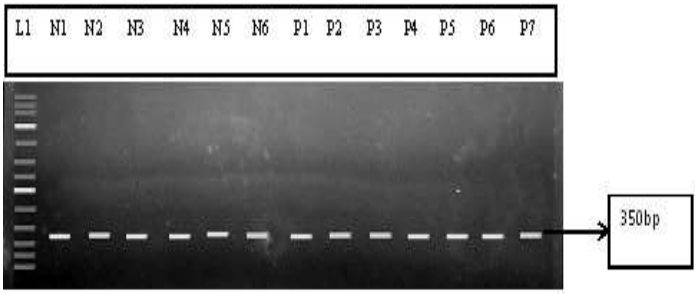

Figure 5: Electropherogram of 2% agarose gel showing amplified products of 350 bp by conventional primer OCT-F3 and OCT-R3.

The lane L1 represents 100 bp ladder while lanes P1 to P3 represents AML patients, lanes P4 to P7 represents ALL patients and lanes N1 to N6 represents control group.

Amplification of *OCT4* gene was done in normal and AML patients with bisulfite specific primers after bisulfite treatment. All the samples show the bands on amplification after bisulfite genomic treatment. This indicates the conversion of unmethylated Cytosine to Uracil by bisulfite treatment

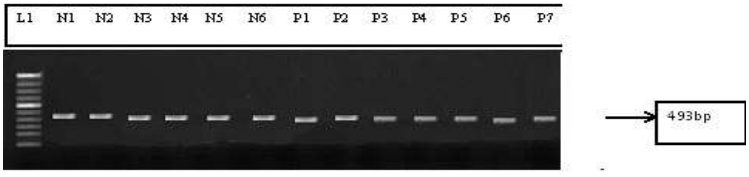

Figure 6: Electropherogram of 2% agarose gel showing amplified products of 493 bp bisulphite specific primers BSP-F1 and BSP-R2 in normal and AML patients.

The lane L1 represents 100 bp ladder while lanes P1 to P7 represents AML patients and lanes N1 to N6 represents control group

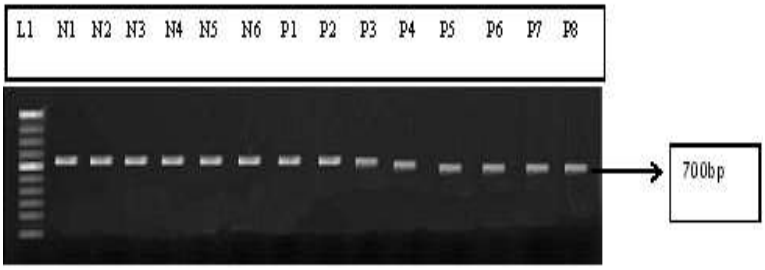

Figure 7: Electropherogram of 2% agarose gel showing amplified products of 700 bp bisulphite specific primers BSP-F2 and BSP-R2 in AML patients.

The lane L represents 100 bp ladder while lanes P1 to P8 represents AML patients and lanes N1 to N6 represents control group.

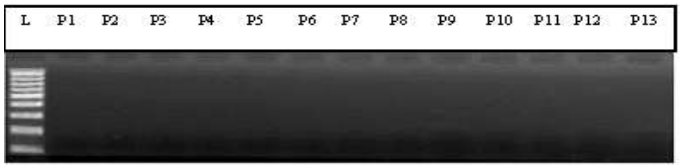

Figure 8: Electropherogram of 2% agarose gel showing no amplified products by conventional primer OCT-F3 and OCT-R3 in AML patient after bisulfite genomic treatment.

The lane L represents 100 bp ladder while lanes P1 to P13 represents AML patients

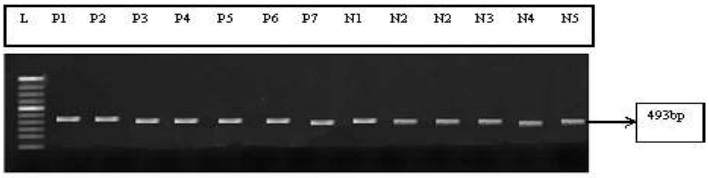

Figure 9: Electropherogram of 2% agarose gel showing amplified products of 493 bp bisulphite specific primers BSP-F1 and BSP-R1 in ALL patients and normal individuals. The lane L represents 100 bp ladder while lanes P1 to P7 represents ALL patients and lanes N1 to N5 represents control group

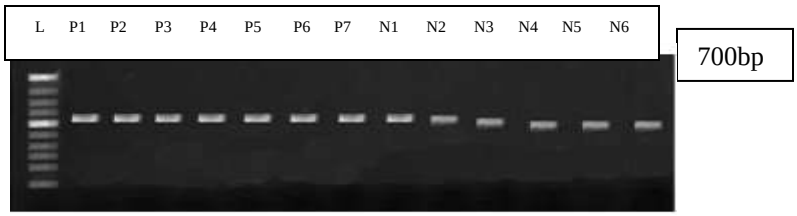

Figure 10: Electropherogram of 2% agarose gel showing amplified products of 700 bp bisulphite specific primers BSP-F2 and BSP-R2 in ALL patients and normal individuals. The lane L represents 100 bp ladder while lanes P1 to P7 represents ALL patients and lanes N1 to N6 represents control group

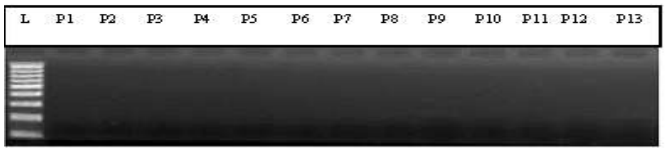

Figure 11: Electropherogram of 2% agarose gel showing no amplified products of 350 bp by conventional primer OCT-F3 and OCT-R3 in ALL patients after bisulfite genomic treatment.

The lane L represents 100 bp ladder while lanes P1 to P13 represents ALL

Discussion

Leukemia is a hematopoietic cancer.[2] Its point of origin is bone marrow. The main causative agents for leukemia are aberrations at gene and chromosomal level. Such injuries at gene level works as oncogene and tumor suppressor genes and has a direct role in onset of leukemia.[2,25] Acute myeloid leukemia (AML) is a heterogeneous disorder and it depends upon various factors such as age, mutational status and karyotype. It is most common in adults and majority of patients could not survive and die within 2 years of disease diagnosis. Acute Lymphoid leukemia is a malignant disease which is associated with the lymphoid initiator cells.[3] It effects both children and adults but the most common in children. Children between 2 to 5 years of age are more susceptible to ALL. 80 % of ALL patients can be cured if disease diagnosed at earlier stages.[26] Epigenetics includes modifications like histone tail modification, chromatin remodeling and DNA methylation within Cytosine residues and other

sites without CpG island.[27] Gene expression is regulated by these epigenetic modifications, these modifications molds the chromatin structure in such a way that the proteins or different transcriptions factors can have access to the chromatin and thus make certain genes on or silence their expression.[28] Epigenetics have a great role in the cell proliferation, differentiation, early stages of embryo development and proper maintenance of many cellular processes.[14]

Methylation in human genome mostly occurs at cytosine residues and particularly at cytosine which are present at CpG island and accomplished by methyltransferases. Human genome contains small patches of CpG sites called as CpG islands. More than 70% of human genome promoter regions contain CpG islands.[14] Methylation of cytosines in CpG island causes transcriptional inactivation by attracting the histone deacetylases and histone methyltransferases that alter the histone structure and convert it into a condensed form. In condensed form enzymes don't have access to promoter region.[29] Once the methylation has been occurred, some of the genes remain methylated and transcriptionally inactive. In cancer cells DNA methylation has been found to be reduced at intergenic regions which usually comprises of methyl-cytosine. Although, hypo- and hyper-methylation can be associated with cancer.[29] In malignant cells decreased pattern of methylation at cytosine residues has been observed.[30]

In the present study the methylation status of *OCT4* gene has been analysed specifically on cytosine residues in the 1500bp 5/ upstream region from the first coding exon. The study was conducted on Acute Myeloid Leukemia and Acute Lymphoid Leukemia. The method used for the analysis of methylation pattern was Bisulfite modification. This method involves conversion of unmethylated cytosine to uracil. OCT 4 gene is active in pre-embryonic stages of the development, when the cells are in differentiating stage and turns off when the differentiation of the cells starts due to methylation mostly at regulatory regions such as promoter, enhancer and silencer. In leukemia due to hypomethylation, this gene is found to be in an active state. The OCT 4 (also known as POU5F1) plays a crucial role in cancer by promoting tumor initiation, growth, metastasis, and therapy resistance. At transcription level *OCT4* expression is regulated by the cis-acting elements which are located upstream of *OCT4* gene and chromatin methylation

The present study was conducted on control group, AML and ALL patients 40 each, to analyse the methylation status of *OCT4* gene. The study was based on WBC count, age and blast cells. From the collected clinical data from patients, it was observed that on average WBCs count in AML and ALL patients were significantly high as compared to normal. The blast cells values which were nil in normal individuals were high in AML and ALL patients. From the data it can be concluded that males are more prone to AML and ALL as compared to females. It was found that *OCT4* was hypomethylated in most of AML and ALL patients. With the help of bisulfite technique, methylation pattern was determined in control, AML and ALL individuals. In AML and ALL patients hypomethylation status of *OCT4* gene were observed after bisulfite genomic treatment when amplified with conventional and bisulfite specific primers and results of amplification confirms the conversion of unmethylated cytosine to uracil.

For more accurate analysis of methylation pattern in AML and ALL, the amplified samples are needed to be sequenced. With the help of sequencing complete methylation pattern can be determined. Further studies are required with the extended sample size to illustrate the exact mechanism of methylation of *OCT4* gene in leukemia and to find the association of this gene with severity of AML and ALL, the complete 5/ prime upstream region and all the regulatory elements in this region are required to analyse.

Conclusion

From the present study it's concluded that *OCT4* is an important transcription factor and has a crucial role in leukemogenesis. It has a vital role in stem cell pluripotency. *OCT4* has been found to be hypomethylated in both type of leukemia. Bisulfite modifications can be used at clinical level to detect AML and ALL. Further investigations are required for understanding the phenomenon of methylation in *OCT4* gene.

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Cite this article as: Tareen MU, Balqees A, Sajid M, Hassan W, Zafar U, Tariq H. Methylation Analysis of Oct 4 Gene in AML and ALL. Mirpur J Med Sci. 2025;3(2): 139-145. https://doi.org/10.65433/mjms.v3i2.104.