Methylation Analysis of SOX2 gene in AML and ALL patients by Bisulfite Medification

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ABSTRACT
Epigenetic modifications play a crucial role in the regulation of gene expression. Methylation is one of the most important players that determines the fate of gene expression and renders a fundamental role in the regulation of stem cell pluripotency. *SOX2* is an important transcription factor that plays a key role in maintaining stem cell pluripotency. Aberrant methylation in the promoter region of *SOX2* disrupts the normal cellular differentiation. The present study aimed to assess the methylation status of CpG islands in the promoter region of *SOX2* gene in AML and ALL patients. This gene is unmethylated in human embryonic stem cells, whereas it is shown to be partially methylated in AML and ALL. Methylation profile of *SOX2* gene was analyzed in 50 samples each of Acute Myeloid Leukemia, Acute Lymphocytic Leukemia and control samples. DNA samples of AML and ALL were modified with sodium bisulfite. Successful completion of bisulfite modification was confirmed by amplifying the bisulfite treated samples by Methylation-specific PCR. Few of the samples showed amplification with methylated primer and others with unmethylated primer, however majority of the patient samples showed partial methylation at the CpG islands of *SOX2* gene. 29 out of 50 ALL patients and 30 out of 50 AML showed amplification with both methylated and unmethylated primers, that represent partial methylation. These findings suggest that aberrant methylation in the promoter region of *SOX2* gene contributes to leukemogenesis. Furthermore, it was found that males are more prone to AML and ALL as compared to females. More studies are required to scrutinize the detailed methylation profile of *SOX2* gene in AML and ALL patients. Aberrant methylation of *SOX2* presents a potent therapeutic target for future therapy of AML and ALL.

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Introduction

Leukemia is a type of cancer that resides in the blood, therefore it is known as blood cancer [1]. This disease causes irregular development of blood cells (immature hematopoietic) in bone marrow from stem cells [2].

Reportedly, global occurrence of leukemia is about 0.01% and death rate is 8 per 100,000 persons annually. It usually persists more in adults as compare to children. Generally men are more prone to leukemia worldwide, as compare to women. It was also seen that whites are more sensitive than blacks to leukemia [1, 3]. Generally acute leukemia develops quickly and almost all newly developed cells turn out to be blasts, and within a week or month it causes destruction[3]. Chronic leukemia is slow growing. In chronic leukemia damage occurs slowly usually over prolonged period. Both types of cells are produced in chronic leukemia.

Leukemia can be lymphoid or myeloid, depends upon which type of bone marrow progenitor cells are affected [4].

Aberrant epigenetic landscapes, including deregulated DNA methylation patterns, are a hallmark of many cancers, including the myeloid malignancies, Epigenetics changes can cause carcinogenesis by the silencing of the some of the important genes. [5, 6]. DNA methylation is one of the most stable modification and it's maintained once occurred, except in some cases such as embryogenesis. Other epigenetic modification such as histone modification are more active biological changes regarding expression regulation of gene [7, 8] but they can also cause gene silencing.

The addition of methyl-group to cytosine which forms 5-methylcytosine in DNA. This causes genetic and epigenetic special effects on cell development, differentiation and carcinogenesis[5, 6]. In mammalian DNA methylation is restricted to cytosine residues followed by the guanosine residue (the CpG dinucleotide). Recent studies showed that methylation of cytosine residues other than those found in CpG dinucleotide repeats can be methylated and their methylation causes regulation of embryonic stem cells. However the most important is methylation of CpG repeats [9].

The Sox (sex-determining region Y-Box) family of proteins includes a host of transcriptional factors that are known to have crucial roles in embryogenesis and development.[10, 11] Members of the Sox family have been reported to regulate a diversity of developmental processes, including the maintenance of pluripotency of embryonic stem cells (ESCs; Sox2), testis determination (Sry), chondrogenesis (Sox5, Sox6, Sox9), as well as the development of the cardiac and lymphoid systems (Sox4), lens (Sox1 and Sox2), neural tissues and the brain (Sox1, Sox3, Sox11, Sox14, Sox21)[12, 13] The biological importance of Sox2 is highlighted by the observations that Sox2 homozygous-null mouse embryos die soon after implantation [14] and mutations of the Sox2 gene have been linked to optic nerve hypoplasia and syndromic microphthalmia in humans [15] Sox2 is believed to work in concert with other ESC proteins, particularly Oct4, to maintain self-renewal and the pluripotency of ESCs [14]. Similar to the other Sox family members, Sox2 binds to DNA in a highly sequence-specific manner [12, 13].

Recent studies have implicated Sox2 in cancer biology. Sox2 has been reported to be highly expressed in a number of solid tumors, including cancers of the prostate [16] stomach [17, 18], breast,[19] colorectum [20] brain [21, 22] and testicles[23] and most of these reports focus on the correlation between Sox2 expression and various clinicopathological parameters. Mechanistic studies investigating the role of Sox2 in cancer cells are relatively scarce, but a few recent publications have provided evidence that Sox2 indeed contributes to tumorigenesis and invasiveness. For instance, Sox2 was found to enhance the migration and proliferation of lung cancer cell lines [24, 25]

Significant efforts have been made to evaluate the methylation pattern of *SOX2* gene in different cancers such as gastric cancer, lung cancer and glioblastomas. This study has been

designed to investigate role of *SOX2* gene in Acute Myeloid Leukemia and Acute Lymphocytic Leukemia. The major objectives of this study were:

- To investigate the role of *SOX2* in hematological malignancies i.e., AML and ALL.
- To analyze methylation pattern of CpG islands in the promoter region of *SOX2* gene.
- To assess the relationship between aberrant methylation of *SOX2* gene and onset of AML/ALL.
- Clinical characterization of patients and determining their methylation profile.

Material and Method Sample collection

The study was conducted on the blood samples of one hundred and fifty samples. This pool of samples included fifty normal individuals, fifty Acute Myeloid Leukemia patients and fifty Acute Lymphocytic Leukemia patients. Blood samples were collected form AML/ALL patients treated at PIMS (Pakistan Institute of Medical Sciences), Islamabad. Prior consent was taken from the Ethical Committee of PIMS. The identification of AML and ALL patients was based on TLC count and bone marrow biopsy. 5 ml 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.

2.3 DNA Extraction

Genomic DNA was extracted from peripheral blood samples by Phenol-Chloroform method (organic method) [26]

Agarose Gel Electrophoresis

The extracted DNA was checked on 1% agarose gel. 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. For tracking of DNA 10 µl of ethidium bromide was added.

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. 1000ml of 1X TBE (100ml 10 X TBE and 900ml distilled water) buffer was poured in the gel tank to carry out electrophoresis at 90 volts for one and an half hour. The gel was illuminated under UV trsansilluminator (Biometra, Gottingen, Germany) to visualize the DNA and gel picture was captured with the help of digital camera DC 290 (Kodak, New York, USA).

2.4.2 Genomic DNA Quantification

Genomic DNA was quantified by taking the optical density (OD) at 260 nm in Generay UV-photometer (Biometra, Goettingen, Germany) and subsequently diluted to 40-50 ng/ul for polymerase chain reaction.

Bisulfite Treatment

There are two methods for bisulfite treatment.

- Commercially Available Kits.

- Conventional method.

We have used EpiJET Bisulfite Conversion Kit (Thermo Scientific cat.no K1461 for bisulfite genomic treatment and the protocol was followed as per the user manual of the manufacturer.

Methylation analysis through Methylation specific PCR.

Methylation specific PCR (MSP) was performed after performing bisulfite modification on AML and ALL samples, to identify the methylation pattern on promoter region. Primers used for MSP are shown in table 1.

Table.1. Primer sequence

Primer		**Sequence**	**Tm**	**Product size**
Methylated	F	5′GTTTATTTATTTTTTTCGA AAAGGC3′	56°C	254bp
	R	5′ATAAATTTCTAACGACCA ATCAACG3′	59.2°C	
Unmethylated	F	5′TGTTGTTTATTTATTTTTT TTGAAAAGGT3′	57.7°C	
	R	5′AATAAATTTCTAACAACC AATCAACAC 3′	59.2°C	

RESULTS

CpG islands in *SOX2* promoter

The upstream 600 bp region of *SOX2* gene was surveyed for the presence of CpG islands using cpgplot software The criteria used was as follows: GC content > 50% and island size > 100 bp.

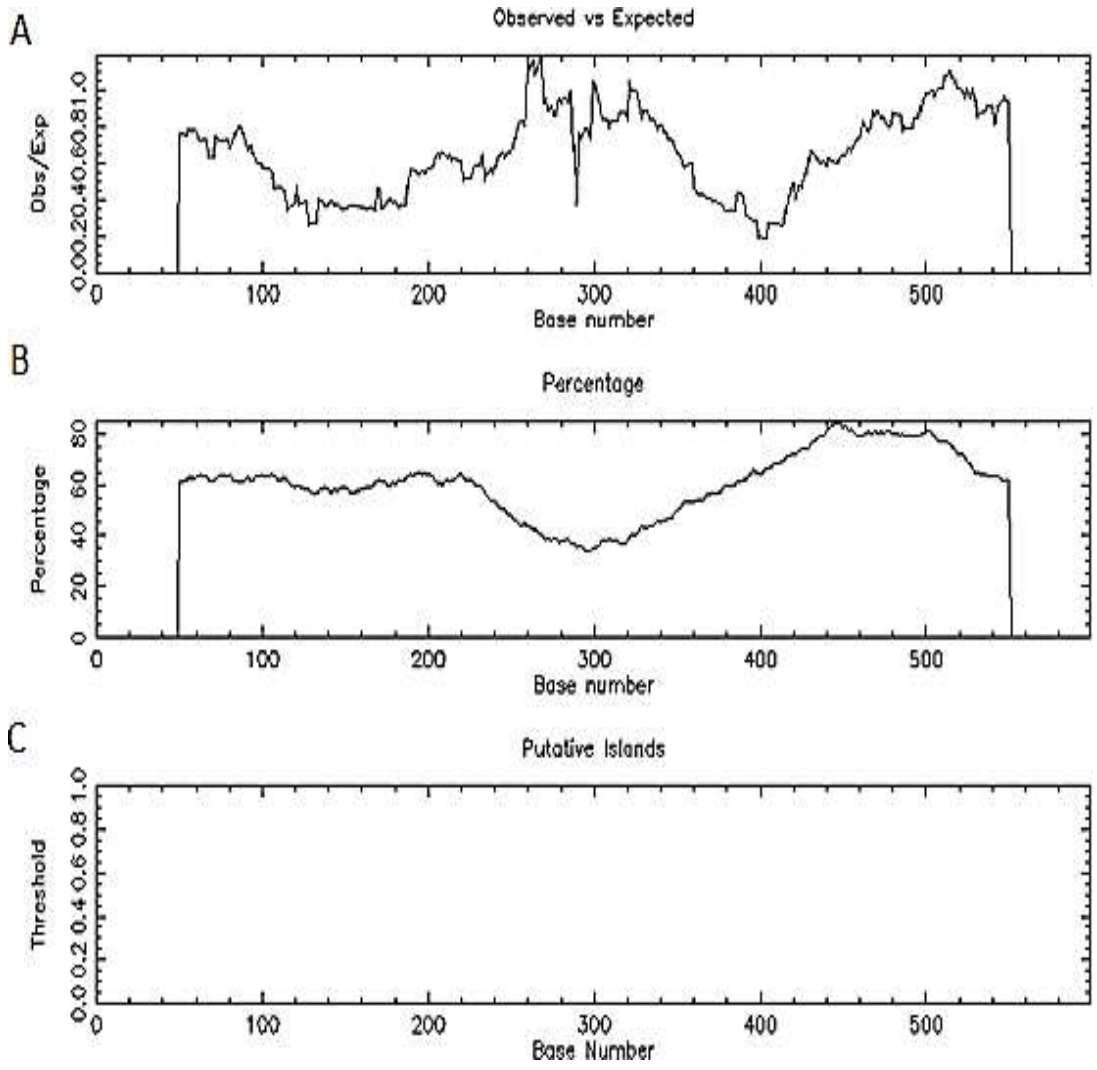

Figure 1. Bioinformatic analysis of *SOX2* promoter. (A) represents the observed/expected ratio of CpG sites (B) represents the GC content as a percentage of the total (C) represents the putative site of CpG island within the -600bp of *SOX2*. (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/).

Analysis of clinical parameters for Acute Myeloid Leukemia

Mean WBC count of AML samples was significantly higher than that of control samples. Low standard deviation in control samples shows that WBC count is close to the average. High standard deviation in AML samples shows that WBC values are scattered from the mean because of elevated WBC count in such patients. T-test confirmed that WBC count in AML patients was significantly high ($P<0.05$) as compared to that of control group. P value obtained from Chi square test was statistically significant that shows significant difference between the two variables i.e., WBCs count of control and AML samples. The data is shown in Table 2. Graph plotted between WBCs count of AML and control samples is shown in figure 2

Analysis of clinical parameters for Acute Lymphocytic Leukemia

Mean WBC count of ALL samples was significantly higher than that of control samples. Low standard deviation in control samples shows that WBC count is close to the average, whereas high standard deviation in ALL samples shows that WBC values are scattered from the mean because of elevated WBC count in such patients. T-test confirmed that WBC count in ALL patients was significantly high ($P<0.05$) as compared to that of control group. P value obtained from Chi square test was statistically significant that shows significant difference between the WBCs count of ALL and control samples. The data is shown in Table 3. Graph plotted between WBCs count of ALL and control samples is shown in figure 3.

Table 1. Statistical parameters of WBC count of control and AML samples

Statistical Parameters	Control WBC Count	AML WBC Count
Mean	7625.98 (±467.96)	12583.68 (±2115.62)
Standard Deviation	3308.529	14957.41
P value (T-test)	0.0287 ($P<0.05$)	
Chi square (χ^2)	228842,49 ($P<0.0001$)	

Table 3. Statistical parameters of WBC count of control and ALL samples

Statistical Parameters	Control WBC Count	ALL WBC Count
Mean	7625.98 (±467.96)	18706.6 (±6476.79)
Standard Deviation	3308.529	45790.9
P value (T-test)	0.0007 ($P<0.05$)	
Chi square (χ^2)	334734,49 ($P<0.0001$)	

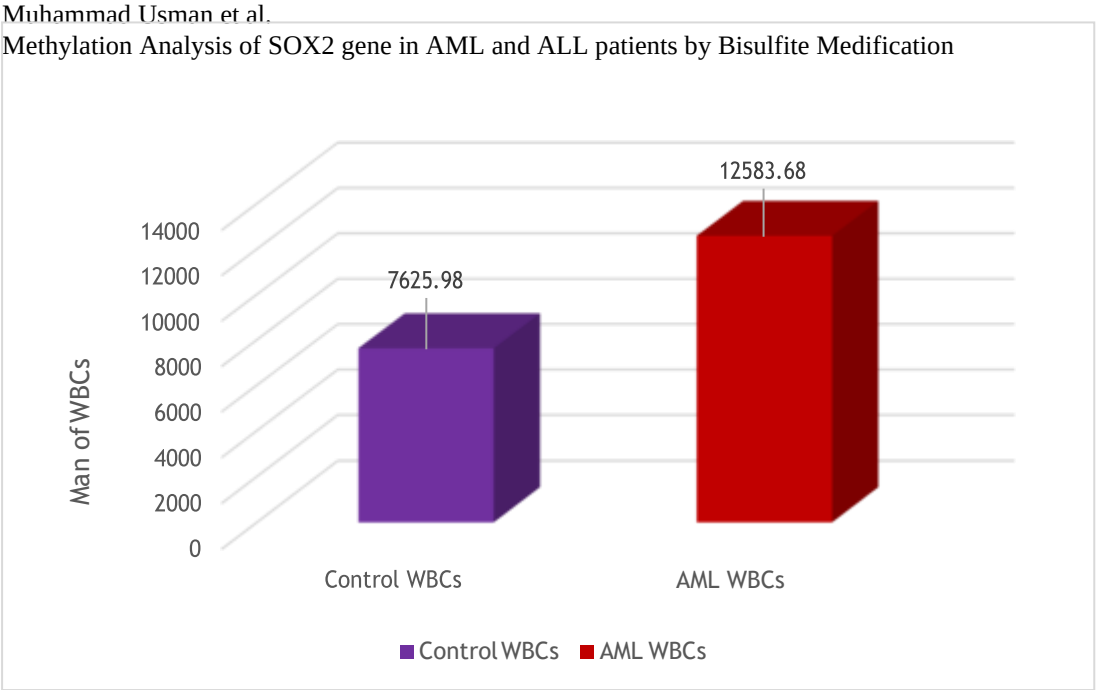

Figure 2. Graph showing comparison of WBCs count in control and AML samples.

Figure 3. Graph showing comparison of WBCs count in control and ALL samples.

DNA amplification of AML, ALL and control Samples

50 control samples, 50 ALL samples and 50 AML samples were amplified by Methylation- specific PCR using two sets of primers i.e., methylated and unmethylated primers of *SOX2*. Samples were successfully amplified, showing band either with methylated primer or unmethylated primer or both. Some samples did not show any band because of failed bisulfite conversion. Details of methylation are shown in Table 3.6. Methylation profile of AML, ALL and control samples is

shown in figure 4.

Table 3.6 Methylation profile of samples amplified by Methylation-specific PCR

Samples	Methylation	Unmethylation	Partial Methylation
Control	4	30	13
AML	2	16	30
ALL	6	13	29

Amplification of *SOX2* gene with methylated and unmethylated primers

Amplification of *SOX2* gene was done in normal, AML and ALL samples with methylated and unmethylated primers after bisulfite treatment. The results of amplification are shown in figures 4 and 5.

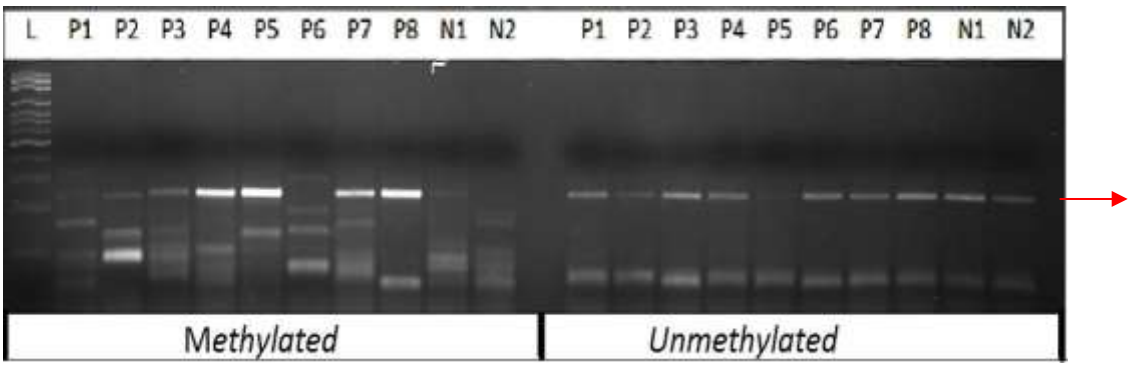

Figure 4. Electropherogram of 2% agarose gel of PCR products stained with ethidium bromide. L represents 100bp ladder. P1 to P4 represent ALL samples, P5 to P8 represent AML Samples and N1 to N2 represent normal samples. All the samples are showing bands except P6 amplified with methylated primer.

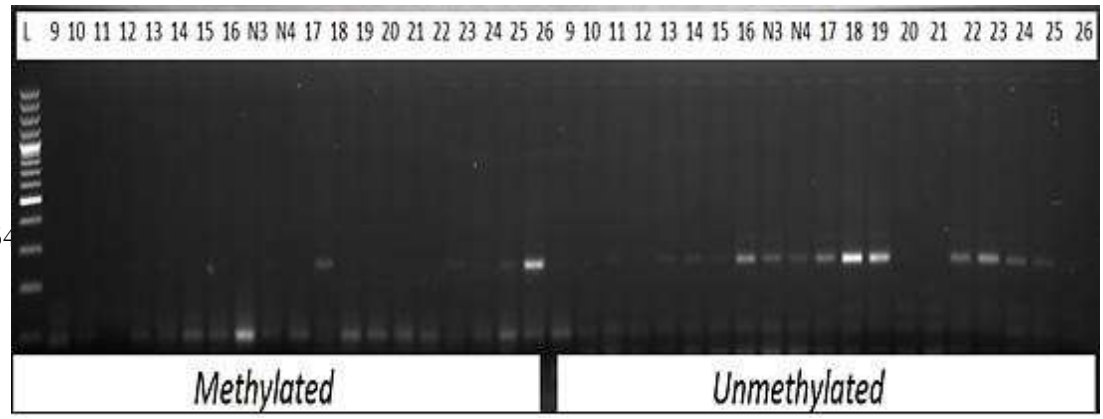

Figure 5. Electropherogram of 2% agarose gel of PCR products stained with ethidium bromide. L represents 100bp ladder. P9 to P16 and P21 to P26 represent ALL samples, P17 to P20 represent AML samples and N3 to N4 represent normal samples. Some of the samples show amplification with methylated primer, some with unmethylated primer and some with both methylated and unmethylated primers. Some samples did not show any bandwith both primers because of incomplete or failed bisulfite conversion.

DISCUSSION

In the present study, methylation status of *SOX2* gene in AML and ALL was evaluated. This study aimed to assess the role of aberrant methylation of *SOX2* gene in the onset of AML and ALL. AML and ALL are hematological malignancies characterized by abnormal number of blood cells. *SOX2* is a self-renewal transcription factor and maintains pluripotency in embryonic stem cells [27, 28]. *SOX2* has been described as an oncogene and its improper functioning is implicated in numerous types of cancer. It controls the physiology of cancer cells by sustaining cancer stem cells and stimulating oncogenic signaling[29] . *SOX2* is involved in a variety of cancers by promoting cellular proliferation (breast, pancreatic, prostate and cervical cancers) [30-32] evading apoptotic signals (gastric cancer, prostate cancer and NSCLC) [31, 33, 34] and promoting metastasis, invasion and migration (colorectal, melanoma, glioma, ovarian, gastric cancer and hepatocellular carcinoma) [35-38] *SOX2* increases CSC markers in pancreatic, ovarian and lung cancer, but research has proven its self-renewal function as well [33, 39] . *SOX2* has been reported to mediate self-renewal in gastric, breast, ovarian, glioma, prostate cancer, lung adenocarcinoma, osteosarcoma and NSCLC [21, 38, 40-42]. This makes *SOX2* an important investigatory gene for its role in various cancers. The role of *SOX2* in many cancer types has been fully investigated, however its role in hematological malignancies remains elusive.

SOX2 is expressed differently in different cell types and it relates with epigenetic modifications, especially methylation. For instance, *SOX2* is normally expressed in gastric mucosa, however, it is downregulated in some gastric carcinomas. This downregulation correlates with aberrant methylation. *SOX2* has been shown to act as tumor suppressor gene in gastric cancer[43]. In contrast, *SOX2* overexpression can also lead to the development of many malignancies, like breast cancer [33, 41]. Epigenetic modifications of *SOX2* play crucial role in regulating its expression. For instance, aberrant methylation of *SOX2* is associated with the onset of malignant gliomas. *SOX2* has been shown to be hypomethylated in malignant gliomas

[38]. Hence, aberrant methylation of *SOX2* gene plays significant role in various cancers.

Therefore, it is important to evaluate the methylation status of promoter region of *SOX2*. Promoter region of *SOX2* is important because majority of CpG content is concentrated over translational initiation codon and upstream of transcriptional start site. Promoter of *SOX2* gene is unmethylated in human embryonic stem cells. Therefore, epigenetics is the major player in *SOX2*-associated carcinogenesis [33]. This makes *SOX2* a potential target for therapeutic intervention.

Epigenetics play important role in regulating the gene expression that is crucial for normal cell functioning. However, epigenetic deregulation contributes to cancer progression. Aberrant methylation (hypomethylation or hypermethylation) of various genes is linked with the onset of leukemia. Various malignancies are characterized by promoter hypermethylation which suggests the role of epigenetic alterations in cancer progression. For instance, gene encoding p15 undergoes hypermethylation and plays a role in hematological malignancies such as MDS and Acute Myeloid Leukemia [44, 45]. Hypomethylation of various genes has also been shown to be associated with leukemogenesis. For instance, *MOK* gene is hypomethylated in Acute Myeloid Leukemia and hence overexpressed[46]. In this study, methylation profile of *SOX2* in AML and ALL was highlighted that still remains obscure.

Methylation profiling of *SOX2* gene depicted that it is in partially methylated state in AML and ALL patients. Some of the patient's samples showed amplification with methylated primer and some with unmethylated primer. However, most of the samples showed amplification with both sets of primers which confirms that *SOX2* is partially methylated at its promoter region. 60% AML and 58% ALL samples showed mix methylation with both methylated and unmethylated primers. These findings suggest that CpG methylation is between 1 to 99%, i.e., not fully methylated or unmethylated. Furthermore, it was observed that males are more prone to AML and ALL as compared to females. These findings correlate with the clinical parameters because most of the patients were undergoing chemotherapy that is based on the administration of demethylating drugs and some patients were newly diagnosed. Methylation density was also found to be correlated with advanced disease. Moreover, band intensity was also directly related to the strength of methylation. This study proved that *SOX2* is partially methylated and contributes to the progression of AML and ALL. However, some post- therapy patients showed unmethylation. These findings are consistent with the fact that carcinogenesis is associated with decrease in methylation. This intermediate methylation is associated with less stability during successive cell divisions for maintaining the methylation density. During carcinogenesis, active demethylation contributes to the conversion of a highly methylated DNA to partially demethylated DNA. This cancer-associated DNA demethylation affects transcription, posttranscriptional processing of transcripts and causes increased recombination [47].

To determine the detailed methylation pattern of *SOX2* gene in leukemia, sequencing or restriction digestion is required. Furthermore, there is a need to analyze methylation pattern of *SOX2* in initially diagnosed leukemic patients by increasing the pool of samples so as to determine the precise methylation profile of *SOX2* prior to therapy. Further studies highlighting the complete methylation status of *SOX2* gene in hematological malignancies and its correlation with gene expression are needed in this regard.

Conclusion

From this study, it is concluded that aberrant methylation of *SOX2* contributes to the progression of AML and ALL and has a critical role in leukemogenesis. *SOX2* has been found to be in partially methylated state in AML and ALL patients. Furthermore, increased frequency of methylation is correlated with advanced disease. Aberrant methylation of *SOX2* presents a potential therapeutic target to effectively treat leukemia in future. Further studies are required to assess the detailed methylation status of this gene and its association with leukemia.

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