Epigenetic Analysis of OCT4 Gene in Blood Cells of Normal and Leukemic Patients

Touseef Rehan[1*], Riffat Tahira[2], Tabassum Rehan[3], Ayesha Bibi[4], Amir Ali Khan[1]

[1]Department of Biochemistry and Molecular biology, Quaid-i-Azam University, Islamabad, Pakistan

[2]Plants Genetic Resources Program, National Agriculture Research Center Islamabad, Pakistan

[3]Department of Biotechnolgy, Quaid-i-Azam University, Islamabad, Pakistan

[4]Department of Genetics, Hazara University, Mansehra, Pakistan

*Corresponding Author: touseefbiochem@gmail.com

ABSTRACT

Epigenetic changes are one of the many causes of cancer. Our research interest lie in the alteration of methylation in the CG in the promotor of OCT4 in the DNA of ALL(acute lyphoblastic Leukaemia). We examined the alteration of methylation of CG at three CCGG loci in the promotor of Oct4 by restriction digestion of CCGG using MSPI and HPaII followed by PCR to amplify the region flanking the CCGG. This simple yet effective methology was used to assess the alteration of methylation in the CG of specific CCGG in the 60 control and 50 ALL patients. CCGG sequence at 2 loci was selected in the 2.7 Kb upstream regulatory region of Oct4 gene. The DNA from the ALL patients were digested with MSPI and HpaII followed by PCR using the primers that flank the CCGG. The PCR bands were observed in the control while they were absent in the DNA of ALL patients indicating the alteration of methylation at these CGs. Our results demonstrate that the specific CG in the CCGG inside the promoter of OCT4 undergoes demthylation in the ALL patients compared to normal. However, research is needed to confirm our preliminary findings.

Key words: Alteration of methylation, Leukaemia, digestion of CCGG, PCR

1. Introduction

Any mechanism that can regulate the genome without changing the primary nucleotide sequence of DNA is epigenetic mechanism. In humans the main epigenetic modification is the methylation of cytosine residue in the dinucleotide sequence CpG [1]

The most important epigenetic tag of embryonic stem cells is that methyle group is covalently attached to 5 C of cytosine ring in CpG dinucleotides[2]. Recent studies suggest that cytosin residues other than those present in CpG dinuceotide sequences, can be methylated and methylation of these cytosine residues is important in regulation of embryonic stem cells. However CpG methylation plays an important role to repress the transcription of transposons and repeat elements. It is also very important in genomic imprinting and X chromosome inactivation [3].

Methylation of Cytosine residued in CpGs can block the expression of certain genes by several ways. First the transcription factors can't recognize and bind to DNA if the specific CpGs are methylated [4]. Secondly the presence of methyl group can enhance the recruitment of certain other proteins also. For example methyl CpG binding protein 2 (MeCp2) will preferentially bind to the methylated DNA and it further enhance the recruitment of histone deacetylases etc. Further deacetylation of histone proteins will enhance the chromatin condensation and repress the transcription, as the access of transcription factors will be blocked [5].

De Novo methylation of DNA and its maintenance is carried out by a family of enzymes, DNA methyl transferases. DNMT3A and DNMT3B carry out de novo methylation during embryogenesis, while it is maintained after embryogenesis by DNMT1b [6]. About 90% of CpGs are methylated at the 5 position of Cytosine ring in mammalian genome. CpG dinucleotides are not uniformly distributed in human genome. For example there are certain regions in human genome which are CpG rich, called CpG islands [7]. Usually in normal tissues these CpG islands are unmethylated and frequently span promoter, untranslated region and exon 1 of most of the genes. If the cytosine residues in the CpG islands are in an unmethyated state, than chromatin configuration will be open, so that the transcription of those particular genes will occur if the transcription factors

will be available. If the CpG islands will be located outside of the upstream 5K region than their methylation will not affect the transcription of RNA [8].

There may be hypomethylation or hypermethylation of DNA occurs due to which cancer will be developed. Tumorigenesis can be progressed by global hypomethylation of DNA. This hypomethylation occurs at various sequences of the genome, including CpG poor promoters, repititive elements, retrotansposons, introns and gene desserts [9]. Genomic instability is increased by hypomethylation of repetitive sequences of DNA, which in turn leads to chromosomal rearrangments. Retrotransposons can be activated and translocated to other regions due to hypomethylation which also leads to genomic instability [10]. Genomic instability due to hypomethylation can cause many diseases, like facial anomalies syndrome and immunodeffeciency [11]. Similarly DNA hpomethylation and genomic instability are invovolved in causing a variety of human cancers. Many growth promoting genes are activated by hypomethylation and leads to development of, such as MAPSIN and R-Ras are activated in gastric cancer, S-100 is activated in colon cancer and MAGE in melanoma [12].

DNA hypermethylation is also associated with te development of a variety of cancers by supressing the expression of tumour supressor genes. Rb, P16, MLH1 and BRCA1 undergo silencing due to hypermethylation and enhance the development of cancer [13].

Embryonic stem cells have two distinctive properties, self-renewal and proliferation. There are some common features shared by embryonic stem cells and cancer cells. It has been reported previously that some important factors of stem cells have been activated in cancer cells. In embryonic stem cells there were 13 genes set that is the signature of these cells. These genes were divided into 3 groups in the previous studies, active core factor genes (SOX2, OCT4 and NANOG), which are expressed in embryonic stem cells, PRC targets and Myc targets. When the expression of these genes was tested in patients of cancer, embryonic stem cells specific genes were highly expressed in poorly differentiated tumors [14].

Recent studies suggest that OCT4 plays a vital role in tumorigenesis also. It has been reported that in cancer cell lines it is reactivated. In drug resistant prostate cancer

the expression of OCT4 was checked and its expression was high in those patients [15].Small population of cells having stem cells like properties was found in adenocarcinoma cells. These are cancer stem cells [16]. OCT4 plays a very important role in maintaining the stemness of these cells. OCT4 is involved in regulation of differentiation and cell growth. Normally it is expressed in pluripotent cells but its strong expression is used as tool to diagnose embryonic carcinomas. An oncogenic role has been proposed for OCT4 in cancer biology [17].

2. Materials and Methods

2.1. Sample collection

Blood samples from 50 normal and 60 leukemic patients of Pakistan were collected. Patient's name, gender ethnicity and type of cancer were recorded.

2.2. Genomic DNA extraction from human blood

Extraction of Genomic DNA from normal and leukemic blood samples was carried out using phenol-chloroform standerd procedure. Quantity of DNA was assessed using Nanodrop 1000 spectrophotometer. Absorbance at 260nm and 280 nm was checked. Absorbance at 260nm/absorbance at 280nm of all the samples were in the range of 1.8 to 2.2, which shows the significant amount of DNA, to use it for digestion with restriction enzymes.

2.3. Treatment with Restriction enzyme specific for CCGG Sequence

Genomic DNA was treated with methylation specific restriction enzymes (Msp1 & HpaII) to cut the CCGG sequence in the promoter of the OCT4 gene.

According to the manufacturer protocol enzymes were used to digest the DNA. The protocol adapted was to make a reaction mixture, which include,

15 µl nuclease free water

2.5 µl 10X Buffer

2.5 µl DNA

3 µl enzyme

The samples were placed in incubator at 37C for 1 hour.

Digested DNA samples analysis was performed on agarose gel of concentration 1.5% through electrophoresis at 90 volts. UV transilluminator (BIO-RAD, Italy) was used for visualization of digested DNA bands. Primer Pairs

GAGGGACGCACGATGAAG, CTCAGACATCTAATACCACGGTAG and GAGGGACGCACGATGAAG, CTACCGTGGTATTAGATGTCTGAG were usedto amplify the upstream region of OCT4 gene.

2.4. Polymerase chain reaction (PCR)

Conventional PCR was carried out to

3. Results

Genomic DNA of normal and leukemic samples was digested with Msp1 and HpaII restriction enzymes. This enzyme can recognize the CCGG sequence and can cut it, whether it is methylated or unmethylated. The electropherograms showed a smear on the gel which is an indication of the fragmentation of whole genomic DNA. Genomic DNA was also digested with HpaII. Electropherogram showed multiple bands on the agarose gel. This is an indication of digestion of DNA with this enzyme. After treatment of genomic DNA extracted from both normal and leukemic samples polymerase chain reaction was carried out for all these samples using primer pair 1 and 2. When PCR was done for normal and leukemic DNA samples digested with restriction enzyme MspI, no bands appear on the gel, which confirms

presence of CCGG in our desired region (Fig1).

Than to assess the methylation at our target cytocine residues methylation specific restriction enzyme HpaII was used to digest the DNA. Then PCR was carried out using two pairs of primers. We found bands on the gel, so our desired region is amplified, which indicates the methylation of our targeted cytosine residue. All the normal samples are showing bands with both the primers pairs. As shown in figure 2 and 3.

Results of PCR using both pairs of primers, after digestion of leukemic samples with HpaII shows that all the samples did not showed any band on the gel, suggesting that internal cytosine residue is unmethylated (Fig4). As HpaII can cut only unmethylated cytosine so after cutting our desired region is not amplified.

Fig 1. Results of PCR for normal and leukemic samples digested with Msp1

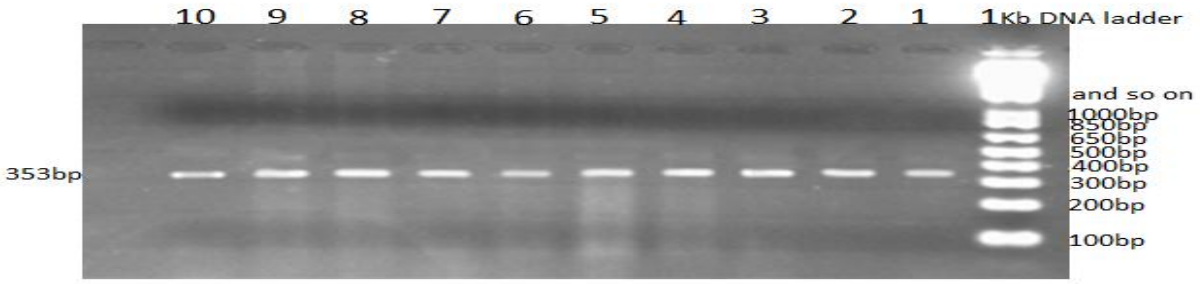


Fig 2. Results of PCR for normal patients samples digested with HpaII restriction enzyme amplified with primer pair 1

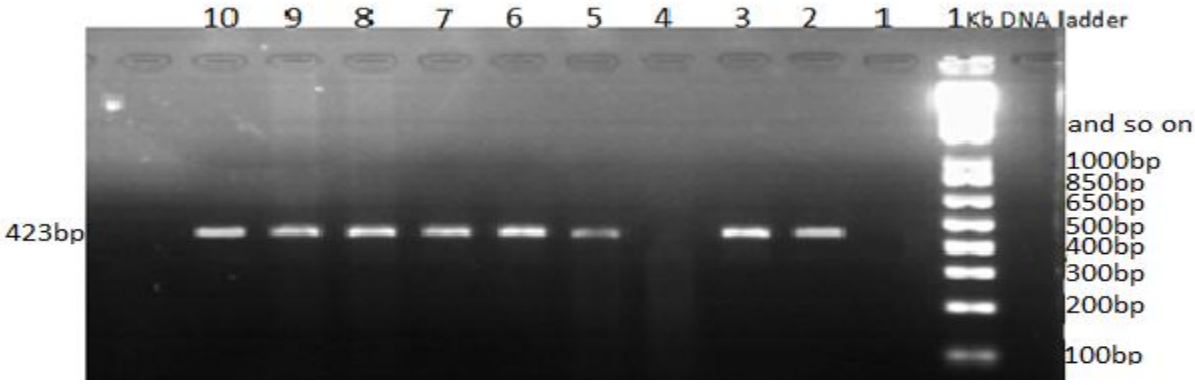
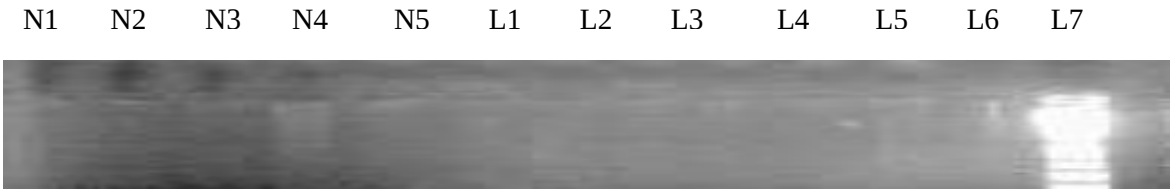


Fig 3. Results of PCR for normal patients samples digested with HpaII restriction enzyme amplified with primer pair 2



4. Discussions

Our study showed that the reactivation of OCT4 gene in case of leukemia is not dependent on the type of leukemia, because we found that unmethylation at our target cytosine residues occur in almost all the five types of leukemia, that are a part of our study. Also we have concluded that the reactivation of this gene does not depend on the age of the patient in case of leukemia, but in patients of acute lymphoblastic leukemia we have found that in patients with older age the methylation at our target cytosine residues is same as in normal samples and in patients of younger age methylation is removed.

OCT4 also known as POU5F1 is a very important transcription factor to maintain the pluripotency of embryonic stem cells. Ectopic expression of OCT4 in epithelial tissues mediates dyplastic growth. In germ cells it has been described as a marker for tumorigenesis [18]. and also it has been reported to be reactivated in cancer cell lines but some other studies shows no up regulation of OCT4 gene in cancer cells [19]. There are some discrepancies raised about OCT4 gene expression, which may be due to 2 protein isoforms of this gene. Its 2 isoforms are OCT4A and OCT4B. The latter of which have no biological function [20]. There also exist some pseudogens of OCT4 gene in humans and mouse, due to which some false positive results can be raised in PCR detection [21]. One of the pseudogene *POU5f1B, residing on chromosome* 8q24, encodes a protein, which has 95% homology with OCT4A. In prostate cancers this pseudogene is also amplified frequently [22]. In the upstream 2Kb region of OCT4 gene there are three regulatory elements, promoter, proximal and distal enhancer. In the early stages of development a specific methylation pattern was observed at the upstream region of OCT4 gene. The primary targets for methylation are proximal enhancer and distal promoter regions, while the distal enhancer and proximal promoter regions have low methylation level. One possibility for high methylation level of proximal enhancer and distal promoter region is the recruitment of enzymes to these sites [23].

A previous study about the role of OCT4 gene in cancer progression shows that ectopic expressions of OCT4 in normal cells of breast will initiate tumor formation in these cells [24]. OCT4 expression is also shown to maintain the cancer stem cells like properties in cancer cells of lungs [25]. Studies on prostate cancer cells also show that common properties of stem cells and cancer cell lines of prostate include over expression of OCT4 gene [26]. Recent studies on ovarian cancer also show that over expression of OCT4 plays a very important role in ovarian cancer progression, recurrance and drug resistance. Over expression of OCT4 gene is also important in maintaining ovarian cancer stem cells properties [27].

5. Conclusion

Our study of cancer epigenome shows that there occur changes in the methylation pattern of regulatory elements of OCT4 gene in all the 5 types of leukemia. OCT4 is a developmentally important gene, encoding a protein that is an important transcription factor for the activation of some other important genes in development. It is active in embryonic stages, while in adults this gene is switched off. Our results show that the methylation of cytosine residues in the upstream region of this gene has been removed in leukemic patients. These results were compared with the methylation pattern of OCT4 gene regulatory elements in normal samples. This provides a clear clue about the unmethylation in leukemic samples.

5. References

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