

## ABERRANT PROMOTER REGION HYPERMETHYLATION OF *HMLH1* GENE IN ESOPHAGEAL CANCER PATIENTS OF KASHMIR VALLEY

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**ABSTRACT:** Introduction: *The Study was a Case Control undertaken to understand the etiology of esophageal cancer in the population of Kashmiri origin. Esophageal cancer is one of the most prevalent cancers in Jammu and Kashmir region of India and has multi-factorial etiology involving dietary habits, genetic factors, and gene environmental interactions. In this study it was attempted to identify whether the altered methylation status of promoter region of Mismatch Repair (hmlh1) gene could be used as a molecular marker associated with esophageal carcinogenesis. Materials and Methods: Aberrant promoter region hypermethylation plays an important role in the progression of esophageal carcinoma. For evaluating the status of hmlh1 promoter region hypermethylation and its association with Esophageal Cancer, methylation specific polymerase chain reaction (MS-PCR) was used. A total of 70 esophageal tissue specimens comprising 50 histopathologically confirmed tumor tissues and 20 histopathologically confirmed normal corresponding tissues as controls were collected for analysis in this study. DNA was extracted and treated with sodium bisulfite which converts unmethylated cytosines to uracil and does not affect methylated cytosines. The modified DNA was amplified in MS-PCR reaction by using methylated and unmethylated promoter specific primers. The MS-PCR products were run on 3% agarose and bands were visualized under UV light. Results: It was found that the frequency of promoter region hypermethylation of mismatch repair gene (hmlh1) in esophageal cancer cases was 56% and in histopathologically confirmed Normals it was 15%. Statistically the association of promoter region hypermethylation of mismatch repair gene (hmlh1) with esophageal cancer was found significant ( $p < 0.05$ ). Observing similar level of hmlh1 promoter hypermethylation in patients with Esophageal Cancer in this high risk region and comparing it with other parts of the world could support the hypothesis that a common molecular mechanism might be involved in tumorigenesis of Esophageal Cancer. As regards promoter*

*hypermethylation status of mismatch repair gene hmlh1 shows a significant increase in promoter region hypermethylation of esophageal cancer patients of Kashmiri origin as compared to controls was observed.*

Keywords—*Esophageal cancer, hmlh1, Hypermethylation, Mismatch repair, MS-PC*

### I. INTRODUCTION

The Study was a Case Control undertaken to understand the etiology of esophageal cancer in the population of Kashmiri origin. This case control study was designed to assess the relationship of promoter Hypermethylation of Mismatch Repair Gene mutl homolog 1 (*hmlh1*) with esophageal cancer. Cancer is currently the cause of 12% of all deaths worldwide. In approximately 20 years of time, the number of cancer deaths annually will increase from about 6 million to 10 million. Cancer prevalence in India is estimated to be around 2.5 million, with over 8, 00, 000 new cases and 5, 50, 000 deaths occurring each year due to this disease in the country (1). Cancer is a complex disease characterized by multiple genetic and epigenetic genomic alterations (2-4). Esophageal cancers are typically carcinomas which arise from the epithelium, or surface lining, of the esophagus (5). Esophageal cancer ranks sixth among all cancers worldwide, with 400 000 new cases being diagnosed per year. This malignancy exists in two principal forms, each possessing distinct pathological characteristics: Esophageal squamous cell carcinoma (ESCC), which occurs at high frequencies in many developing countries, particularly in Asia, and Esophageal adenocarcinoma (EAC), which is more prevalent in Western countries, with a rapid rate of increase in recent years (6). Esophageal carcinoma (EC) is one of the most common cancer occurring globally (7) and is a major cause of cancer related deaths in India. The high incidence areas in India includes North-East India (8) and Kashmir valley (9) where environment and dietary habits play an overwhelming role in the development of EC over

the genetic factors. The annual incidence of esophageal cancer in Kashmir is reported as 42 and 27 for men and women, respectively per 100,000 individuals (9). In Kashmir a lot of dietary features and life style are peculiar, e.g., consumption of hot salted tea, sun-dried vegetables of Brassica family (Hakh), pickled vegetables (Anchar), dried fish, red chillies, spice cakes etc. These food items have been found to contain substantial amount of N-nitroso compounds including N-nitrosopipercolic acid, mono and demines of methane and ethane, with several unidentified nonvolatile N-nitroso compounds (10-13). Methylation changes in mismatch repair (MMR) genes affect their function and result in accumulation of damage leading to genomic instability (14, 15). The MMR system maintains genomic integrity by correcting replicative errors. Generally, it is accepted that defects in MMR genes are responsible for the microsatellite instability (MSI) observed in different diseases including cancer. Point mutations within the MMR genes seem to be infrequent; however, promoter Hypermethylation has been suggested as the main cause of MMR gene silencing (16, 17). Deficiencies in this system result in mutation rates 100-fold higher than those observed in normal cells (18, 19). Human mutl homologue or *hmlh1* is a member of the mismatch repair system whose function is to replicate the genome faithfully (20). Methylation of the *hmlh1* gene has been correlated with the loss of protein expression in ovarian, hepatocellular, endometrial, colorectal and gastric carcinomas in humans (21). There are controversial reports of its association with esophageal cancers (22, 23). The Study was a Case Control undertaken to understand the etiology of esophageal cancer in the population of Kashmiri origin. The Hypermethylation of the promoter region of mismatch repair gene *hmlh1* was not well documented in esophageal cancer population of Kashmiri origin. So this study was confined to study the promoter region Hypermethylation of *hmlh1* gene in esophageal cancer population of Kashmiri origin. In this study a candidate gene approach was used to study a key cancer gene (*hmlh1*) undergoing epigenetic inactivation in esophageal cancer. In this study, it was demonstrated how one single type of DNA alteration, aberrant methylation of gene promoter, can point to pathway disrupted in esophageal cancer.

## DNA METHYLATION AND GENE REGULATION

The regulation of eukaryotic gene expression is a complex process. Transcription initiation is a highly controlled and integrated event that involves *cis*-acting and *trans*-acting factors. The *cis*-acting elements are DNA sequences that act as the substrate for the *trans*-acting factors, and the DNA in the vicinity is prepared for transcription. Increased methylation in the promoter region of a gene leads to reduced expression, whereas methylation in the transcribed region has a variable effect on gene expression.

Several mechanisms have been proposed to account for transcriptional repression by DNA methylation. The first mechanism involves direct interference with the binding of specific transcription factors to their recognition sites in their respective promoters. Several transcription factors, including AP-2, c-Myc/Myn, the cyclic AMP-dependent activator CREB, E2F, and NFκB, recognize sequences that contain CpG residues, and binding of each has been shown to be inhibited by methylation.

The second mode of repression involves a direct binding of specific transcriptional repressors to methylated DNA. The DNA methylation signals are analyzed by the methyl-CpG-binding proteins, the target being the 5' methylated CpG sequence. MeCP1 and MeCP2 were the first two protein complexes identified. However, several new proteins have now been identified. They include MBD1, MBD2, MBD4, and Kaiso. MeCP1, MBD1, MBD2, and MBD4 bind to 5mCpG through a motif called the methyl CpG binding domain (MBD). Kaiso, however, is different in mechanism, as it binds through a zinc finger motif. MBD4 is associated with DNA repair, whereas MBD1, MBD2, MeCP2, and Kaiso have been shown to repress transcription both in vitro and in cell culture assays by interacting with histone deacetylase complexes.

DNA methylation can also affect histone modifications and chromatin structure, which, in turn, can alter gene expression. The underlying patterns of methylated cytosines are important in guiding histone deacetylation to certain residues. At present, there are five known proteins that have the methyl-CpG-binding domain, and four of these

(MeCP2, MBD1, MBD2, and MBD3) are implicated in transcriptional repression. Three of these (MeCP2, MBD2, and MBD3) are in complexes (MeCP-2, MeCP-1 and Mi-2, respectively) that contain histone deacetylases. Studies of methylated transfected genes containing binding sites for all four of these methyl-binding proteins have shown at least partial abrogation of transcriptional repression by treatment with the histone deacetylase inhibitor, trichostatin A.

Earlier it was suggested that histone modification was secondary to DNA methylation, but recent studies on fungus revealed that histone modification can on its own commence the process of DNA methylation.

The methylation of lysine in histones by specific histone methylases is also implicated in changes in chromatin structure and gene regulation. A zone of deacetylated histone H3 and methylation of histone H3 at lysine 9 surrounds a hypermethylated, silenced *hMLH1* promoter, which, when unmethylated and active, is associated with acetylated H3 and methylation of histone H3 at lysine 4 position. Inhibiting DNA methyltransferases, but not histone deacetylases, leads initially to promoter demethylation, followed by gene re-expression, and finally to complete histone code reversal.

## II. MATERIALS AND METHODS

**Sample collection:** A total of 70 esophageal tissue specimens comprising 50 histopathologically confirmed tumor tissues and 20 histopathologically confirmed normal corresponding tissues as controls were collected for analysis in this study. Record was maintained of the complete case history of the patients. All samples were surgically resected and were collected at the Department of surgery SMHS, Karan Nagar, Srinagar India. No patient received pre-operative radiation or chemotherapy. Tissue samples were divided into two parts; one part was sent to histopathologically diagnosis and other half was stored at -80oc for molecular investigations. Only histopathologically confirmed cases and controls were included for molecular analysis. Written informed consent was obtained from all the subjects included in the study and was carried out in accordance with the principles of the Helsinki Declaration. Extraction of genomic DNA: For the isolation of genomic DNA, kit based

method was used. The kit used was Quick-g dnatminiprep supplied by ZYMO RESEARCH. The DNA eluted was stored at 40C for a short time but the vials were kept at -20oc for longer duration storage for further investigation. The integrity of the genomic DNA was examined by gel electrophoresis using 1 % agarose gel and visualized under UV illumination. The quantity of the above isolated esophageal cancer and histopathologically confirmed normal genomic dnas was determined by measuring optical density (OD) at 260nm and 280nm by double beam spectrophotometer (Spectron 2206). Bisulfite treatment of DNA and methylation-specific PCR: DNA methylation patterns in the cpg islands of promoter region of gene was determined by chemical treatment with sodium bisulfite and subsequent MSP as described by Herman *et al.*, 1996 (24). The above extracted Genomic DNA was treated with sodium bisulphite solution under carefully controlled conditions by which unmethylated cytosines are converted by deamination into uracil, but methylated cytosines are resistant to the reagent. This was done by an EZ DNA Methylation –Direct TM Kit supplied by ZYMO RESEARCH.

**Methylation-Specific polymerase chain reaction (MS-PCR):** All the cytosines in the unmethylated product were converted to thymines after bisulfite treatment and amplification, suggesting that the *hmlh1* gene is unmethylated. However, the cytosines in the cpg dinucleotides of methylated product remained unchanged, as methylated cytosines cannot be modified by bisulfite, which indicated that the cpg islands of the gene are methylated. The methylation status of the *hmlh1* gene was analyzed by methylation-specific PCR (MSP) approach, as described by Herman JG. *Et al.* 1996 (24). The bisulfite-treated DNA was used as a template for PCR to amplify. The primers used for amplification of promoter region of gene were listed in the literature along with PCR-annealing temperatures, fragment sizes, and No. Of PCR cycles (25-28) and are shown in Table I. For methylation-specific polymerase chain reaction (MSP) the total reaction volume was 25 µl containing 50–100 ng of bisulfite modified DNA, 20 pmol of each primer, 25 mm dntp s, 1 U Taq Polymerase, 1X PCR buffer (Bangalore Genei, Bangalore). PCR reactions were started by denaturation at 950C for 5 min, followed by 30

cycles at 94°C for 30 s, 60°C (wild type), 60°C (for unmethylated *hmlh1*), 58°C (for methylated *hmlh1*) for 30 s, and 72°C for 30 s, with a final extension at 72°C for 4 min. DNA from normal lymphocytes was used as negative control for methylated alleles of *hmlh1*, and placental DNA treated in vitro with SssI methyltransferase (New England Bio Labs, Beverly, MA, USA) was used as positive control for methylated alleles of *hmlh1*. 15 µl of PCR products were analyzed by electrophoresis on a 3% agarose gel or 8% polyacrylamide gel and visualized by ethidium bromide staining. Data analysis and statistics: The 2-test with Odds ratio was used to examine the differences in the distribution of hypermethylation in promoter region of gene between cases and controls. ORs with 95% CI were computed using unconditional logistic regression using Graph Pad Prism Software Version 5.0 by Graph Pad Software 2236, Avenida de la Playa, La Jolla, CA. Hypermethylation in Cancer: Reports of hypermethylation in cancer far outnumber the reports of hypomethylation in cancer. There are several protective mechanisms that prevent the hypermethylation of the CpG islands. These include active transcription, active demethylation, replication timing, and local chromatin structure preventing access to the DNA methyltransferase. To date, numerous genes have been found to undergo hypermethylation in cancer. The genes that are susceptible are the genes involved in cell cycle regulation (*p16<sup>INK4a</sup>*, *p15<sup>INK4a</sup>*, *Rb*, *p<sup>14ARF</sup>*) genes associated with DNA repair (*BRCA1*, *MGMT*), apoptosis (*DAPK*, *TMS1*), drug resistance, detoxification, differentiation, angiogenesis, and metastasis.

### III. RESULTS

In this study it was attempted to identify in individuals with esophageal cancer whether the altered methylation status of promoter region of Mismatch Repair (*hmlh1*) gene could be used as a molecular marker associated with esophageal carcinogenesis. For the first time such a study was conducted regarding this Mismatch Repair (*hmlh1*) gene as related to esophageal cancer in the population of Kashmiri origin. The integrity of the genomic DNA was examined by gel electrophoresis using 1% agarose gel and visualized under UV illumination. Analysis of promoter methylation of *hmlh1* was carried out in resected 50 invasive primary Esophageal cancer

cases, respectively. Figure 1 and 2 shows representative examples of MS-PCR results. Among the 50 cases, less than a half exhibited at least one methylated primer amplification (<50%). In other hand, for some cases, we observed both the methylated and unmethylated primer amplification, this can be probably explained by the presence of infiltrating lymphocytes and/or nonmalignant epithelial cells in the primary tumors. It was observed that 28 out of 50 (56%) of the esophageal Cancer cases showed bands in methylated (M) wells or in both wells which confirms that Promoter region of Mismatch Repair Gene (*hmlh1*) gene is hypermethylated (Figure 1: lane 1-4-Case 67 and 68). However 22 out of 50 (44%) of the esophageal cancer cases does not show bands in methylated wells but in unmethylated wells which confirms Promoter region of Mismatch Repair Gene (*hmlh1*) is not hypermethylated (Figure 1: lane 5,6-Case 05) and in positive control (universal methylated DNA) band seen in methylated well only (Figure 1: lane 7,8). In case of histopathologically confirmed esophageal controls it was seen that 17 out of 20 (85%) showed no bands in methylated (M) wells but bands were seen in unmethylated (U) wells which confirms *hmlh1* gene promoter is not hypermethylated (Figure 2: lane 3-6-Control 18 and 06) while in remaining 03 histopathologically confirmed esophageal Controls, bands were seen in methylated (M) wells or in both wells which confirms that remaining 15% of histopathologically confirmed esophageal Controls were hypermethylated (Figure 2: lane 1, 2-Control 67) and in negative control (lymphocyte DNA) band seen in methylated well (Figure 3: lane 7, 8). Statistically the Association of promoter region Hypermethylation of mismatch repair gene (*hmlh1*) with esophageal cancer was found significant with the p value =0.0028 and was evaluated by  $\chi^2$  (Chi square) test with Odds ratio (O.R=7.2121, 95% C.I=1.822-27.79). The Frequency of hypermethylation of promoter region of mismatch repair gene (*hmlh1*) in esophageal cancer cases and controls is tabulated in Table II and represented histogramically in Figure 3.

### IV. DISCUSSION

Esophageal cancer is one of the least studied and deadliest cancers, with a remarkable geographical distribution and a low likelihood of cure. Therefore, the current challenges in the management of esophageal cancer are to obtain a

better understanding of the underlying molecular biological alterations to provide new treatment options. It is well known that esophageal carcinogenesis is a multistage and progressive process which includes basal cell hyperplasia (BCH), dysplasia (DYS), carcinoma in site (CIS) and advanced esophageal carcinoma. A variety of genetic lesions are involved in esophageal carcinogenesis, including gene amplifications, loss of heterozygosity (LOH) or homozygous deletions, mutations, and chromosomal rearrangements. From the above mentioned genetic lesions, mutations are greatly focused on. Synergistic effect of dietary, environmental, genetic and microbial factors is being associated with the development of esophageal cancer and it has multifactorial epidemiology (29-34). The contributing factors are not the same in different populations of the world and a common risk factor is yet to be identified. Majority of the esophageal cancer cases are found in developing countries of the world, particularly in Asia (35, 36). Kashmir is a known region of high incidence of esophageal cancer (37-39) and is part of so called "Asian Esophageal cancer belt". Despite much effort that has been done in improving treatment and diagnosis, esophageal cancer prognosis is still poor, making it the sixth most fatal malignancy in the world (40). DNA methylation in cancer has become the topic of intense investigation. Promoter hypermethylation is an alternative mechanism of gene inactivation in carcinogenesis (41). Several studies have suggested that aberrant methylation of the promoter causes transcriptional silencing of some important suppressor genes, such as p16 (42), E-cadherin (43), and von hippellindau (VHL) gene (44), and this has been implicated in the carcinogenic process in many cancers (41). The *hmlh1* protein, a mismatch repair enzyme, maintains the fidelity of the genome during cellular proliferation. It has no known enzymatic activity and probably acts as a „molecular matchmaker“, recruiting other DNA-repair proteins to the mismatch repair complex (45). Dysfunction of a mismatch repair system such as *hmlh1* and *hms2* could alter microsatellites, short tandem repetitive sequences(46).The mismatch repair system is composed of a highly diverse group of proteins that interact with numerous DNA structures during DNA repair and replication(47).Reports of hypermethylation in cancer far outnumber the reports of hypomethylation in cancer. There are several

protective mechanisms that prevent the hypermethylation of the cpg islands. These include active transcription, active demethylation, replication timing, and local chromatin structure preventing access to the DNA methyltransferase (48).To date, nearly 50% of numerous genes have been found to undergo hypermethylation in cancer. The genes that are susceptible are the genes involved in cell cycle regulation (p16ink4a, p15ink4a, Rb, p14arf) genes associated with DNA repair (*hmlh1*,BRCA1, MGMT), apoptosis (DAPK, TMS1),angiogenesis (THBS1, VHL),invasion (CDH1, TIMP3) ,drug resistance, detoxification, differentiation, and metastasis (49).Considering the important role of promoter methylation in inactivation of *hmlh1* which is one of the frequently altered genes in carcinoma of esophagus and many other human cancers, In this study it was attempted to identify in individuals with esophageal cancer whether the altered methylation status of promoter region of Mismatch Repair (*hmlh1*) gene could be used as a molecular marker associated with esophageal carcinogenesis. For the first time such a study was conducted regarding this Mismatch Repair (*hmlh1*) gene as related to esophageal cancer in the population of Kashmiri origin. To determine the status of Mismatch Repair (*hmlh1*) gene promoter methylation in Esophageal Cancer Cases from the population of Kashmiri origin, we performed Methylation Specific Polymerase chain reaction for *hmlh1* gene in 50 surgically resected esophageal cases and compared with that of 20 histopathologically confirmed normal tissues.

Figure 1 and 2 shows examples of MS-PCR results. The frequency of promoter hypermethylation was 56% (28 out of 50) for *hmlh1* gene in Cases. Among the 50 cases, less than a half exhibited amplifications with methylated primer only (<50%). In other hand, for some Cases, we observed amplifications with both methylated as well as unmethylated primers; this can be probably explained by the presence of infiltrating lymphocytes and/or non-malignant epithelial cells in the primary tumors. When we reviewed the literature, the methylation frequency ranged from 8 to 50% for *hmlh1* (50-52). The higher percentage of *hmlh1* promoter hypermethylation seen in the present study may be because of the difference in type of sample used, promoter region assessed and/or technique employed. In earlier studies paraffin-embedded tissue material was used (50-

52), while in the present study we employed fresh biopsy specimens. Wang *et al.*, 2003 (53) reported a higher percentage of *hmlh1* hypermethylation with *hpaII*-based PCR methylation assay when compared with methylation-specific PCR, which involves bisulfite pre-treatment of DNA. The lower methylation reported in these earlier studies compared with our study may be due to the techniques employed. From the results and data it was observed that 28 out of 50 (56%) of the esophageal cases showed bright bands in methylated (M) lane and diminished or no bands in unmethylated (U) lane which confirms *hmlh1* gene promoter is methylated (Figure 1). However 22 out of 50 (44%) of the cases showed no bands in methylated (M) lane and bright bands in unmethylated (U) lane which confirms *hmlh1* gene promoter is not methylated (Figure 1). In case of normal esophageal controls 17 out of 20 (85%) of the esophageal controls showed no bands in methylated (M) wells and bright bands in unmethylated (U) wells which confirms *hmlh1* gene promoter is not hypermethylated (Figure 2 control 18, 06 and 08) while in remaining 03 controls, bands were seen in methylated (M) wells as well as in unmethylated wells which confirms that remaining 15% of normal esophageal controls were hypermethylated (Figure 2 control 67). Statistically the association of promoter region hypermethylation of mismatch repair gene (*hmlh1*) with esophageal cancer was evaluated using 2-test (chi-square test) with odds ratio and was found

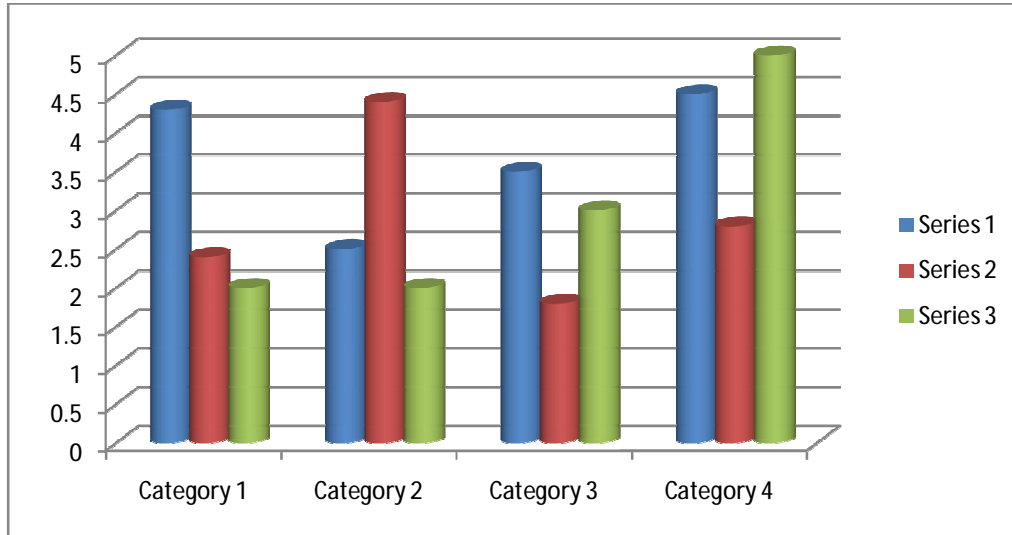
significant ( $p=0.0028$ , odds ratio=7.2121 and 95% C.I=1.822-27.79). This study on Esophageal Cancer showed that more than 50% tissues expressed methylated *hmlh1* promoter. When compared with *hmlh1* methylation data available from other high prevalent regions such as China, Japan, and Iran, the results were comparable with the disease prevalence. Methylation levels were much higher in Caspian littoral of Iran where the prevalence of esophageal cancer is higher than what is in Kashmir or other regions of China and Japan.

## V. CONCLUSION

This study has interestingly revealed that promoter region hypermethylation status of mismatch repair gene *hmlh1* shows a significant increase in esophageal cancer patients of Kashmiri origin as compared to controls. Esophageal carcinogenesis is a stepwise process of the accumulation of genetic and epigenetic abnormalities. It is clear that promoter hypermethylation of *tsgs* is as important for this multistep process as genetic changes in the progression of esophageal carcinogenesis. Our study has supplemented the steadily growing list of genes inactivated by promoter hypermethylation in esophageal carcinoma, these provide not only new insights into the molecular basis of the diseases but also list of interesting candidate genes for the development of molecular markers which might contribute to the improvement of diagnosis and also prognosis.

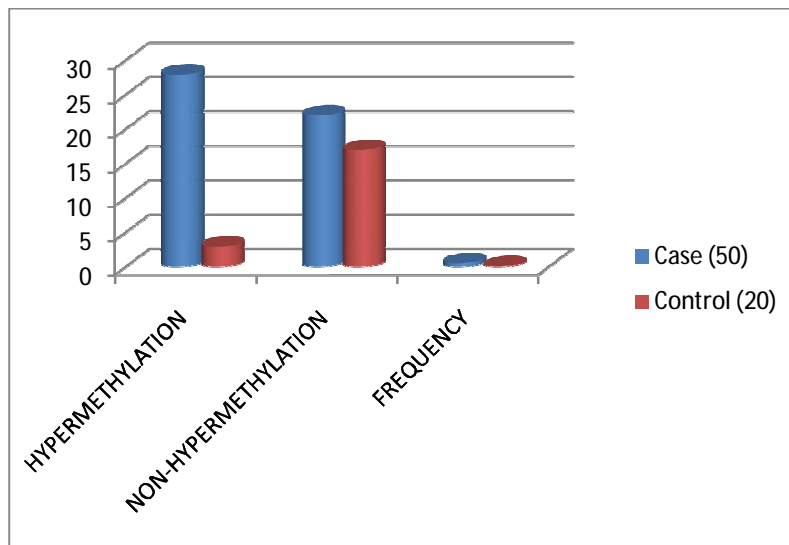
**Table I: Represents the primer sequence of *hmlh1* gene with PCR-annealing temperatures, fragment sizes, and No. of PCR cycles:**

	Sequence (5' 3')		Tm (°C)	Size (bp)	PCR cycles
(U)	F	5'- TTTGATGTAGATGTTTTAT TAGGGTTGT-3'	60	115	30
	R	5'- ACCACCTCATCATAACTACCCACA-3'			
	F	5'-ACGTAGACGTTTTATTAGGGTCGC- 3'	58	110	30
	R	5'-CCTCATCGTAACTACCCGCG-3'			



**Table II: Promoter Region Hypermethylation of Mismatch Repair (*hmlh1*) of Case vs Control as; Total Cases, Hypermethylation, Non-Hypermethylation and Frequency**

PARAMETER	Case (50)	Control (20)
HYPERMETHYLATION	28	03
NON-HYPERMETHYLATION	22	17
FREQUENCY	56%	15%



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