"A Study on Methylation Changes in Mismatch Repair Gene (*hMLH1*) in Esophageal Cancer Patients of Kashmir Valley"

Dissertation Submitted for the Award of the Degree of Master of Philosophy in Biochemistry

By

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UNDER THE JOINT SUPERVISION OF

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CERTIFICATE

Certified that the work embodied in the dissertation entitled **"A Study on Methylation Changes in Mismatch Repair Gene (*hMLH1*) in Esophageal Cancer Patients of Kashmir Valley"** is the bonafide work of Mr. Arif Akbar Bhat and has been carried out under the joint supervision of Dr. Sabhiya Majid (H.O.D Department of Biochemistry, Govt. Medical College Srinagar) and Prof. (Dr) Akbar Masood (H.O.D Department of Biochemistry, University of Kashmir). The work is suitable for the award of M.Phil degree in Biochemistry.

It is further certified that no work under this heading has previously been submitted to the University of Kashmir for the award of any degree or diploma to the best of our belief.

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DECLARATION

I, Arif Akbar Bhat, declare that the work embodied in this dissertation entitled **"A Study on Methylation Changes in Mismatch Repair Gene (*hMLH1*) in Esophageal Cancer Patients of Kashmir Valley"** has been carried out by me in the Department of Biochemistry, Government Medical College, Srinagar (Research Centre University Of Kashmir) and is original. The work embodies the results of my observations which are advancement to the previous knowledge in the subject.

Place: **Arif Akbar Bhat**

Date:

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ABBREVIATIONS

µg	Microgram
µl	Microlitre
A	Adenine
AML	Acute Myeloid Leukemia
BCH	Basal Cell Hyperplasia
bp	Base Pair
BPB	Bromophenol Blue
BRCA1	Breast related cancer antigen I
C	Cytosine
C.I	Class interval
CIS	Carcinoma in Site
cm	Centimeter
CpG	cytosine phosphodiester bond with Guanine
CRC	colorectal cancer
DAPK	Death Associated Protein Kinase
DNA	Deoxyribose Nucleic Acid
DNMT	DNA Methyltransferase
dNTP	Deoxyribose nucleotide Triphosphate
DYS	Dysplasia
e.g.	For example
EAC	Esophageal Adenocarcinoma
EC	Esophageal Cancer
EDTA	Ethylene Diamine Tetraacetic Acid
ESCC	Esophageal Squamous Cell Carcinoma

EtBr	Ethidium Bromide
Fig.	Figure
G	Guanine
GIT	Gastrointestinal Tract
Gm.	Grams
HATs	Histone Acetyltransferases
HDACs	Histone Deacetylases
hMLH1	Human mutL homolog 1, colon cancer, nonpolyposis type
HMTs	Histone Methyltransferases
HNPCC	Hereditary Non-Polyposis Colon Cancer
HP1	heterochromatin protein 1
Hr.	Hours
LOH	Loss of Heterozygosity
M	Molar
MBD	m5CpG-Binding Domain
mg	Milligram
MGMT	o6 methyl guannine methyl transferase
min	Minutes
ml	Mili litre
mM	Mili Molar
MMR	Mismatch Repair
mRNA	messanger Ribonucleic Acid
MSI	Microsatellite Instability
MS-PCR	Methyl Specific polymerase Chain Reaction

MTHFR	Methylene tetrahydro-folate Reductase
NaCl	Sodium Chloride
NCI	National cancer institute
ng	Nanogram
NSCLC	Non-small cell lung cancer
O.D	Optical Density
O.R	Odds Ratio
^{o}C	Degree Celsius
PAGE	Polyacrylamide Gel Electrophoresis
PAHs	Polyaromatic Hydrocarbons
PCR	Polymerase Chain Reaction
pmol	Pico Mole
Rb	retinoblastoma
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
RT	Room Temperature
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SDS	Sodium Dodecyl Sulphate
Sec	Second
T	Thymine
TAE	Tris Acetic Ethylene Diamine Tetraacetic Acid
Taq	*Thermus aquaticus* DNA polymerase

TFs	Transcription Factors
TNM	Tumour Node Metastasis
Tris	Tris (hydroxylmethyl amino methane)
UV	Ultra violet
VHL	Von Hippel Lindau
YSR	Year Survival Rate

Abstract

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. Also further an association of hypermethylation of hMLH1 gene with esophageal cancer in relation to clinicopathological features of Gender and Age was evaluated.

Esophageal cancers are one of the most fatal cancers in the world and are considered to be the eighth most common malignancy. The prognosis of Esophageal Cancer is poor as its symptoms appear in the late stage of the disease. This 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. Genetic abnormalities of proto-oncogenes, tumor suppressor genes and mis-match repair genes have been demonstrated to be involved frequently in esophageal carcinogenesis; chronic inflammation leading to malignancy in the esophagus may be due to errors in mismatch repair (MMR) genes such as hMLH1. Inactivation of the hMLH1 gene expression by aberrant promoter methylation plays an important role in the progression of esophageal carcinoma. In the present study the role of hMLH1 promoter methylation in 50 histopathologically confirmed esophageal cancer tissues and compared it with corresponding histopathologically confirmed Normal adjacent tissues was studied by methylation-specific polymerase chain reaction (MS-PCR).

For evaluating the status of hMLH1 promoter hypermethylation and its association with Esophageal Cancer, a methylation specific polymerase chain reaction (MS-PCR) was used. 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 applying methylated and unmethylated promoter specific primers. Universally methylated DNA was used as positive control and DNA from normal lymphocytes used as negative control. The MS-PCR products were run on 3% agarose and bands were visualized under UV light.

It was found that the frequency of promoter region hypermethylation of mismatch repair gene (hMLH1) in esophageal cancer cases was 56% (28 out of 50) and in histopathologically confirmed normals it was 15% (03 out of 20).Statistically the association of promoter region hypermethylation of mismatch repair gene (hMLH1) with esophageal cancer was evaluated using χ^2-test (chi-square test) with odds ratio and was found significant and the $p<0.05$. It was also found that the methylation status of MutL homolog 1 (hMLH1) gene in esophageal cancer cases was high in Males (60%) compared to Females (47%) and in Controls, Males (16%) also shows high with respect to Females (12%) which shows insignificant association as $p>0.05$.From the data it was concluded that The Frequency of MutL homolog 1 (hMLH1) gene promoter region hypermethylation was found high in Esophageal Cancer Cases of above 40 years of age (56%) and in controls (16%) and was significant as $p<0.05$ compared to below 40 years of age (50%) and in controls (0%) and association was insignificant as $p>0.05$ and was evaluated by Fishers exact test.

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. This became more apparent when the data for hypermethylation was interpreted taking Gender into consideration here it was seen that Males shows higher frequency of promoter region hypermethylation as compared to females which was earlier reported in literature and also patients of above 40 years of age shows high frequency compared to below 40 years of age.

1.1 Introduction to Cancer

Cancer is the uncontrolled growth of abnormal cells anywhere in a body. The abnormal cells are termed cancer cells, malignant cells, or tumor cells. Cancer (medical term: malignant neoplasm) is a class of diseases in which a group of cells display:

- Uncontrolled Growth
- Invasion that intrudes upon and destroys adjacent tissues and
- Sometimes Metastasis or Spreading to other locations in the body via lymph or blood.

These three malignant properties of cancers differentiate them from benign tumors, which do not invade or metastasize. Cancer occurs when a cell's gene mutations make the cell unable to correct DNA damage and unable to commit suicide. Similarly, cancer is a result of mutations that inhibit oncogene and tumor suppressor gene function, leading to uncontrollable cell growth. All cancers begin in cells, the body's basic unit of life. These cells grow and divide in a controlled way to produce more cells as they are needed to keep the body healthy. When cells become old or damaged, they die and are replaced with new cells. However; sometimes this orderly process goes wrong. The genetic material (DNA) of a cell can become damaged or changed, producing mutations that affect normal cell growth and division. When this happens, cells do not die when they should and new cells form when the body does not need them. The extra cells may form a mass of tissue called a Tumor (Figure 1).Cancer is currently the cause of 12% of all deaths worldwide. In approximately 20 years' time, the number of cancer deaths annually will increase from about 6 million to 10 million. The principal factors contributing to this projected increase are the increasing proportion of elderly people in the world (in whom cancer occurs more frequently than in the young), an overall decrease in deaths from communicable diseases, the decline in some countries in mortality from cardiovascular diseases, and the rising incidence of certain forms of cancer, notably lung cancer resulting from tobacco use (National Cancer Control Programmes, 2002). Cancer prevalence in India is estimated to be around 2.5 million, with over 8, 00,000 new cases and 5,50,000deaths occurring each year due to this disease in the country (Nandakumar, 1996).Cancer is a complex disease characterized by multiple genetic and epigenetic genomic alterations (*Jones and Baylin, 2002;* Feinberg *et.al.*, 2006;Esteller, 2007).Many cancers and the abnormal cells that compose the cancer tissue are further identified by the name of the tissue that the abnormal cells originated from for example:

- Breast Cancer
- Colon Cancer
- Lung Cancer
- EsophagealCancer etc.

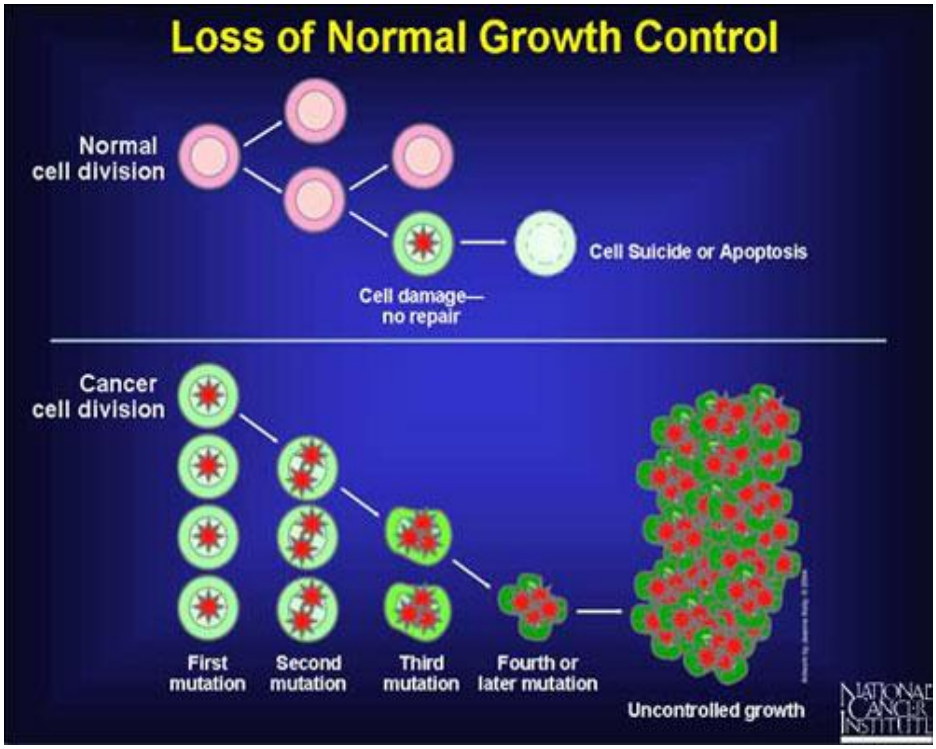

Figure 1: Loss of normal growth control in cancer cells.

(Source- National Cancer Institute)

1.2 Introduction toEsophageal Cancer (EC)

Esophageal cancer (or oesophageal cancer) is malignancy of the esophagus. Esophageal cancers are typically carcinomas which arise from the epithelium, or surface lining, of the esophagus (*Esophageal cancer,* Mount Sinai Hospital).Esophageal cancer is a relatively rare form of cancer, but some world areas have a markedly higher incidence than others: Belgium, China, Iran, Iceland, India, Japan, the United Kingdom, appear to have a higher incidence, as well as the region around the Caspian Sea (Stewart *et al.,* 2003). Esophageal cancer ranks sixth among all cancers worldwide, with 400 000 new cases being diagnosed per year. This cancer is the eighth most commonly occurring cancer and the sixth most common cause of cancer death in the world. It occurs most often in men over 50 years old. 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 (Stewart *et al.,* 2003)(Figure 2). A general rule of thumb is that a cancer in the upper two-thirds is a squamous cell carcinoma and one in the lower one-third is a adenocarcinoma. Rare histologic types of esophageal cancer are different variants of the squamous cell carcinoma, and non-epithelial tumors, such as leiomyosarcoma, malignant melanoma, rhabdomyosarcoma, lymphoma and others (Shield *et al.,* 2005; Halperin*et al.,* 2008).

Prognosis depends on the extent of the disease and other medical problems, but is fairly poor, because most patients present with advanced disease. By the time the first symptoms such as dysphagia start manifesting themselves, the cancer has already well progressed. The overall five-year survival rate (5YSR) is approximately 15%, with most patients dying within the first year of diagnosis (Polednak, 2003). Staging is a careful attempt to find out whether the cancer has spread and, if so, to what parts of the body. Knowing the stage of the disease helps the doctor plan treatment. Listed below are descriptions of the four stages of esophageal cancer.

- Stage I. The cancer is found only in the top layers of cells lining the esophagus.
- Stage II. The cancer involves deeper layers of the lining of the esophagus, or it has spread to nearby lymph nodes. The cancer has not spread to other parts of the body.

- Stage III. The cancer has invaded more deeply into the wall of the esophagus or has spread to tissues or lymph nodes near the esophagus. It has not spread to other parts of the body.

- Stage IV. The cancer has spread to other parts of the body.

Esophageal cancer can spread almost anywhere in the body, including the liver, lungs, brain, and bones.Esophageal carcinoma (EC) is one of the most common cancer occurring globally (Parkin*et al.,* 2005) and is a major cause of cancer related deaths in India. The high incidence areas in India includes North-East India (Phukan*et al.,* 2001) and Kashmir valley (Khuroo*et al.,* 1992) where environment and dietary habits play an overwhelming role in the development of EC over the genetic factors. The main cause appears to be a combination of environmental, dietary and genetic factors. Kashmir is one of the three provinces (Jammu, Kashmir, and Ladakh) of the Jammu and Kashmir state. There is high incidence of gastrointestinal malignancies in general and esophageal cancer in particular in Kashmir valley and constitute more than 15% of all cancer cases in Kashmir. The valley is considered a part of "Esophageal cancer belt" in Asia and has unique socio-cultural and dietary features that make it a very intriguing region for studying this disease. The annual incidence of esophageal cancer in Kashmir is reported as 42 and 27 for men and women, respectively per 100,000 individuals (Khuroo*et al.,* 1992).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 chilies, spice cakes etc. These food items have been found to contain substantial amount of N-nitroso compounds including N-nitrosopipecolic acid, mono and diamines of methane and ethane, with several unidentified nonvolatile N-nitroso compounds (Kumar*et al.*, 1992; Siddiqi*et al.*, 1992, 1998).

Gastrointestinal (GI) cancers account for about 20% of all cancers worldwide. In the global incidence of cancer, gastric cancer is the second, colorectal cancer is the third, and esophageal cancer is the sixth most common tumor (Chan and Rashid, 2006).In neoplasms, including GI cancers, epigenetic changes play a key role in the process of tumorigenesis (Baylin , 2002, 2006).

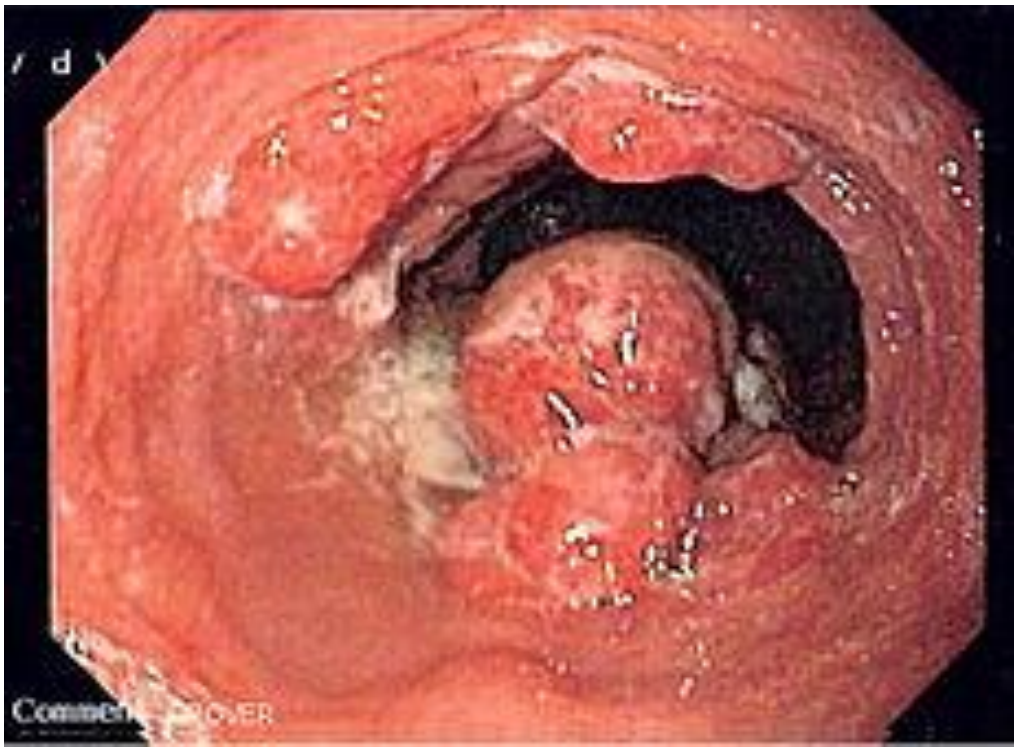

Figure 2: Endoscopic image of patient with esophageal adenocarcinoma seen at gastro-esophageal junction

(Source: Esophageal cancer Wikipedia)

1.3 Introduction to Epigenetics

The term *epigenetic* refers to information which is transmitted from the parental genome to the next generation of cells which is not encoded by the primary DNA sequence. Epigenetic mechanisms are essential for the regulation of gene expression and genome integrity in normal cells (for reviews see (Bird, 2002; Li, 2002; Jaenisch and Bird 2003). Epigenetic information is often transmitted by methylation of the 5 carbon position of cytosine within a CpG dinucleotide, also referred to as DNA methylation. CpG dinucleotides are under-represented in the genome, but over-represented in short regions of 500– 4,000 bp (base pair) in length, known as CpG islands, which are rich in CpG content (Bird, 2002; Takai and Jones 2002). CpG islands are present in the proximal promoter regions of about 60% of the genes in the mammalian genome and are, generally, unmethylated in normal cells. Patterns of DNA methylation and chromatin structureare profoundly altered in neoplasia and include genome wide losses of and regional gains in DNA methylation (Feinberg and Vogelstein 1983).Global hypomethylation was shown to cause genomic instability which in turn may promote secondary genetic alterations (Eden *et al*., 2003; Gaudet*et al*., 2003), whereas local hypermethylation of promoter regions is associated with transcriptional silencing and can lead to the loss of tumor-suppressor gene function. The molecular mechanisms of epigenetic silencing during tumor formation are only partially understood. In normal cells the pattern of CpG methylation is brought about by a group of enzymes known as the DNA methyltransferases (DNMT). The DNMTs known to date are DNMT1, DNMT1b, DNMT1o, DNMT1p, DNMT2, DNMT3A, DNMT3b with its isoforms and DNMT3L (Robertson *et al.,* 2002). Methylation can be de novo (when CpG dinucleotides on both DNA strands are unmethylated) or maintenance (when CpG dinucleotides on one strand are methylated). DNMT1 has de novo as well as maintenance methyltransferase activity, and DNMT3A and DNMT3b are powerful de novo methyltransferases (Marzo*et al*., 1999; Robertson et *al.,* 2000; Costello and Plass 2001). Methylation of various biological molecules including DNA is dependent on S-adenosylmethionine (SAM), the principal biological methyl group donor (Stern *et al.,* 2000; Yi *et al.,* 2000). Considering the critical role of methylation in various cellular processes, it is understandable that any alteration in the availability of SAM may have a profound effect on cellular growth, differentiation and function in both health and illness. Methylenetetrahydrofolate reductase (MTHFR), a rate-limiting enzyme in the conversion of homocysteine to methionine, which is the substrate for

SAM, may play a regulatory role in the folate metabolism and methylation process (Bolander, 2002).

Elevated homocysteine levels, either due to MTHFR mutation or a diet deficient in cofactors for the methylation cycle, result in the formation of S-adenosyl L-homocysteine, which is a competitive inhibitor of SAM. This, as a result, affects methylation in various biological processes (Figure 3).

CpG islands, frequently located at the 5′-end regulatory regions of genes, are subject to epigenetic modifications including DNA methylation and histone modification that are known to play an important role in regulating gene expression (Jones and Baylin 2002). In normal cells, the majority of promoter CpG islands are protected from this epigenetic event and thus they are unmethylated. Conversely, in cancer cells, several promoter CpG islands are hypermethylated and form a closed repressive chromatin configuration that affects the transcription initiation of the corresponding genes (Estelle*r et al.,* 2001,2002; Baylin, 2006).

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 (Clark and Melki 2002).

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 ($p16^{INK4a}$, $p15^{INK4a}$, *Rb*, p^{14ARF})
- The genes associated with DNA repair (*hMLH1*,*BRCA1*, *MGMT*)
- The genes involved in apoptosis (*DAPK, TMS1*)
- The genes associated with angiogenesis (*THBS1, VHL*)
- The genes involved in invasion (*CDH1*, *TIMP3*)
- The genes involved in drug resistance, detoxification, differentiation, and metastasis (Jones and Baylin 2002).

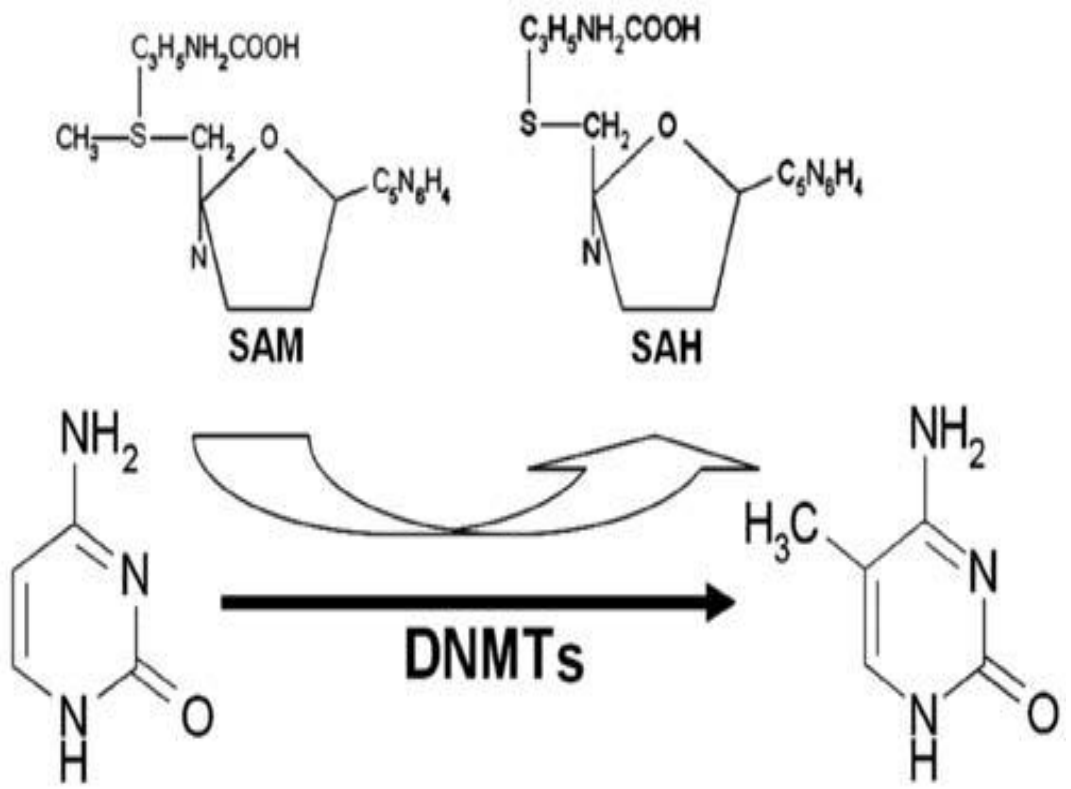

Figure 3: Cytosine gets converted into 5'methyl cytosine by the action of S-adenosylmethionine (SAM) which donates methyl groups and is converted to S-adenosylhomocysteine (SAH) in presence of DNMTs.

(Source: Review article on role of methylation)

1.4 Introduction to *hMLH1*Gene

The DNA mismatch repair (MMR) protein *hMLH1* is encoded by the MutL homolog 1 (*hMLH1*) gene in humans and is a homologue of the DNA MMR gene mutL of Escherichia coli (Figure 4). The MMR function is associated with DNA replication, to correct for deficiencies in DNA polymerase proofreading function. A missing gene or mutations of this gene and other MMR genes (MSH2, MSH6, or PMS2) leads to microsatellite instability (MSI) and this dysfunction is highly associated with hereditary non-polyposis colon cancer (HNPCC or Lynch syndrome) (Bronner*et al.*, 1994).The *hMLH1* gene provides instructions for making a protein that plays an essential role in DNA repair. This protein fixes mistakes that are made when DNA is copied (DNA replication) in preparation for cell division. The *hMLH1* protein joins with another protein, the PMS2 protein, to form an active protein complex. This protein complex coordinates the activities of other proteins that repair mistakes made during DNA replication. The repairs are made by removing a section of DNA that contains mistakes and replacing the section with a corrected DNA sequence (Jascur and Boland 2006).Human MutL homologue or *hMLH1* is a member of the mismatch repair system whose function is to replicate the genome faithfully (Ahuja*et.al*., 1998). Deficiencies in this system result in mutation rates 100-fold higher than those observed in normal cells (Thomas*et al.*, 1996; Jiricny, 2006).

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. Also further an association of hypermethylation of *hMLH1*gene with esophageal cancer in relation to clinicopathological features was evaluated as Gender and Age.

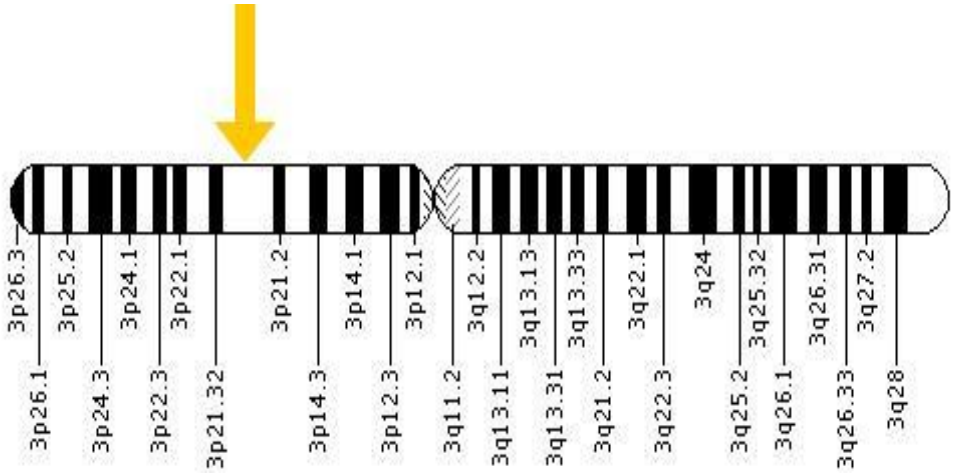

Figure 4: Location of *hMLH1* gene on short p arm of chromosome 3

(Source: Handbook Genetics home reference)

REVIEW OF LITERATURE:

2.1 Literature about General Cancer

The body is made up of many types of cells. These cells grow and divide in a controlled way to produce more cells as they are needed to keep the body healthy. When cells become old or damaged, they die and are replaced with new cells. However, sometimes this orderly process goes wrong. The genetic material (DNA) of a cell can become damaged or changed, producing mutations that affect normal cell growth and division. When this happens, cells do not die when they should and new cells form when the body does not need them. The extra cells may form a mass of tissue called a tumor. Cancer (medical term: malignant neoplasm) is a class of diseases in which a group of cells display uncontrolled growth, invasion that intrudes upon and destroys adjacent tissues, and sometimes metastasis, or spreading to other locations in the body via lymph or blood. These three malignant properties of cancers differentiate them from benign tumors, which do not invade or metastasize. Cells can experience uncontrolled growth if there are damages or mutations to DNA, and therefore, damage to the genes involved in cell division. Four key types of gene are responsible for the cell division process: oncogenes tell cells when to divide, tumor suppressor genes tell cells when not to divide, suicide genes control apoptosis and tell the cell to kill itself if something goes wrong, and DNA-repair genes instruct a cell to repair damaged DNA. Cancer occurs when a cell's gene mutations make the cell unable to correct DNA damage and unable to commit suicide. Similarly, cancer is a result of mutations that inhibit oncogene and tumor suppressor gene function, leading to uncontrollable cell growth. All cancers begin in cells, the body's basic unit of life. These cells grow and divide in a controlled way to produce more cells as they are needed to keep the body healthy. When cells become old or damaged, they die and are replaced with new cells. However, sometimes this orderly process goes wrong. The genetic material (DNA) of a cell can become damaged or changed, producing mutations that affect normal cell growth and division. When this happens, cells do not die when they should and new cells form when the body does not need them. The extra cells may form a mass of tissue called a Tumor.

Cancer is currently the cause of 12% of all deaths worldwide. In approximately 20 years' time, the number of cancer deaths annually will increase from about 6 million to 10 million. The principal factors contributing to this projected increase are the increasing proportion of elderly people in the world (in whom cancer occurs more frequently than in the

young), an overall decrease in deaths from communicable diseases, the decline in some countries in mortality from cardiovascular diseases, and the rising incidence of certain forms of cancer, notably lung cancer resulting from tobacco use (National Cancer Control Programme 2002).Of the 10 million new cancer cases seen each year worldwide, 4.7 million are in the more developed countries and nearly 5.5 million are in the less developed countries. Although the disease has often been regarded principally as a problem of the developed world, more than half of all cancers occur in the developing countries. In developed countries, cancer is the second most common cause of death, and epidemiological evidence points to the emergence of a similar trend in developing countries (Stewart and Kleihues 2003).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 (Nandakumar, 1996).Cancer is a complex disease characterized by multiple genetic and epigenetic genomic alterations (*Jones and Baylin 2002*; Feinberg *et.al.*, 2006; Esteller, 2007). DNA methylation is one of the most important epigenetic alterations and plays a critical functional role in development, differentiation and diseases (*Jones and Baylin* 2002). Through the activity of DNA methyltransferases (DNMTs), DNA methylation occurs at the cytosine residue in the context of 5'-CG-3' (CpG dinucleotide) across human genome (Laird, 2003). During the developmental process, DNA methylation plays an essential role in X chromosome inactivation in female somatic cells and in the mono-allelic silencing of parentally imprinted genes. Once these DNA methylation patterns are acquired in the early embryo stage, these patterns are inherited and maintained in successive cell generations. Promoter regions are usually enriched with CpG dinucleotides, known as CpG islands; and hypermethylation of these islands correlates with transcriptional silencing of corresponding genes (Herman and Baylin 2003).

2.2 Literature about Esophageal Cancer (EC)

Esophageal cancers are one of the most fatal cancers in the world and are considered to be the eighth most common malignancy (Parkin *et al.*, 2005). The prognosis of EC is poor as its symptoms appear in the late stage of the disease. Also, the treatment of EC is protracted, decreases the quality of life and is lethal in significant number of cases, making it the seventh leading cause of death from cancer (Parkin *et al.*, 2005). There are two main histological types of esophageal cancer; squamous cell carcinoma (SCC) and adenocarcinoma (ADC). Squamous cell carcinoma of the esophagus remains predominantly a disease of the

developing world and adenocarcinoma a disease of western developed societies, associated with obesity and gastro-esophageal reflux disease (GERD). Esophageal cancer is more frequent in men as compared to women (Parkin *et al.,* 2005).Approximately 11,000 to 13,000 new cases of esophageal cancer are diagnosed each year in the United States alone. Hospital-based data on cancer from India indicate that esophageal cancer ranks third in males and fourth in females (Chitra *et al.,* 2004). Genetic, epigenetic and environmental factors including diet have been implicated in the causation of this cancer (Mayne *et al.,* 2001; Yang *et al.,* 2005). The esophagus is a 25-cm-long muscular tube of the digestive tract carrying food from the mouth to the stomach. Patients present with symptoms that include dyspepsia, dysphagia and regurgitation. Chronic injury or irritation and inflammation to the esophagus can arise due to chronic infection, drug use, ingestion of corrosive chemicals, idiopathic or allergic causes, alcohol consumption, smoking, tobacco chewing and tea and coffee consumption (Kim *et al.,* 1997; Vaughan *et al.,* 1998; Li and Yu 2003). More than 30% of the adult population suffers from esophageal disorders like esophagitis, reflux esophagitis due to gastroesophageal reflux disease (GERD), etc (Rosai, 1996). GERD caused by the upward backflow of stomach acid, bile, pepsin, ingested liquids and foods into the esophagus results in reflux esophagitis. Apart from precancerous lesions like hyperplasia, dysplasia and carcinoma in situ, Barrett's esophagus is another inflammatory, premalignant condition of the esophagus, primarily seen to be associated with GERD (Bani *et al.,* 2000; Kagawa *et al.,* 2000).Esophageal cancer is a relatively rare form of cancer, but some world areas have a markedly higher incidence than others: Belgium, China, Iran, Iceland, India, Japan, the United Kingdom, appear to have a higher incidence, as well as the region around the Caspian Sea (Stewart and Kleihues 2003). The American Cancer Society estimates that during 2007, approximately 15,560 new esophageal cancer cases will be diagnosed in the United States (Esophageal Cancer, 2006). Esophageal tumors usually lead to dysphagia (difficulty swallowing), pain and other symptoms, and are diagnosed with biopsy. Small and localized tumors are treated surgically with curative intent. Larger tumors tend not to be operable and hence are treated with palliative care; their growth can still be delayed with chemotherapy, radiotherapy or a combination of the two. In some cases chemo- and radiotherapy can render these larger tumors operable (Enzinger and Mayer 2003). Prognosis depends on the extent of the disease and other medical problems, but is fairly poor, because most patients present with advanced disease. By the time the first symptoms such as dysphagia start manifesting themselves, the cancer has already well progressed. The overall five-year survival rate

(5YSR) is approximately 15%, with most patients dying within the first year of diagnosis (Polednak, 2003).Individualized prognosis depends largely on stage. Those with cancer restricted entirely to the esophageal mucosa have about an 80% 5YSR, but sub mucosal involvement brings this down to less than 50%. Extension into the muscularis propria (muscular layer of the esophagus) has meant a 20% 5YSR and extension to the structures adjacent to the esophagus results in a 7% 5YSR. Patients with distant metastases (who are not candidates for curative surgery) have a less than 3% 5YSR.

The epidemiology of esophageal cancer is characterised by remarkable differentiation in incidence against geographical distribution and ethnic backgrounds. It is known that Asian countries, especially China and Iran have the highest rates of esophageal cancer in the world. A high risk region represented by the "*Asian esophageal cancer belt*" ranges from northern Iran all the way to north central China passing through Turkmenistan, Uzbekistan and Kazakhstan. Kashmir valley is also considered a part of this belt and the annual incidence of esophageal cancer in Kashmir is reported as 42 and 27 for men and women, respectively per 100,000 individuals (Khuroo *et al.*, 1992). Esophageal cancer has a multifactorial epidemiology and a synergistic effect of dietary, environmental, genetic, epigenetic and microbial factors is being associated with its development (Zhou *et al*., 1999; Langergren *et al*., 2000; Nayar *et al*., 2000; Shi *et al*., 2000; Zhang *et al*., 2000; Dhillon *et al.*, 2001). The contributing factors are not the same in different populations of the world and a common risk factor is yet to be identified. The suspected risk factors for EC in Kashmir are broadly classified into environmental factors (including diet) and genetic factors.

Environmental factors known to play important roles in the etiology of human cancers include chemical carcinogens (e.g., cigarette smoke), dietary contaminants (aflatoxin B1/AFB1) and physical carcinogens (ionizing and UV radiation). Lifestyles such as smoking, alcohol consumption, excess exposure to sunlight, fat consumption and stress may also contribute to cancer development (Peto, 2001; Mathers, 2004).There is ample evidence of an association between DNA methylation and environmental influences like exposure to viruses (e.g., liver (Shen, 2002), and stomach (Kang, 2002) and diet (e.g., folate) (Waterland and Jirtle 2003) in both normal (aged) tissues and cancer.

Many studies have associated the unique dietary habits with the development of esophageal cancer in Kashmir (Khuroo *et al.*, 1992) although; there is no strong scientific support for the same. Nevertheless, the findings of various studies of the presence of the

considerable amounts of *N*-nitroso compounds like *N*-nitroso pipecolic acid; mono-nitroso piperazine; methyl benzyl nitrosoamine; diethyl nitrosoamine and dimethyl nitrosoamine in the local food items have further strengthened the association of such food items in the development of the esophageal cancer in Kashmir (Siddiqi *et al.,* 1992a, 1992b, 1998). The micronutrient deficiencies or excess (vitamins A/C/E, carotenoids, zinc, and copper) may also have a role in esophageal carcinogenesis (Fong *et al*., 1999; Lutz, 1999; Siassi *et al*., 2000; Nazir *et al*., 2008).

2.3 Literature about Epigenetics

The four nucleotide bases of DNA—Cytosine (C), Adenine (A), Guanine (G), and Thymine (T)—form a total of 16 possible dinucleotide pairs. One of these dinucleotides, in which a Cytosine is adjacent to a Guanosine in the 5' direction (the CpG dinucleotide), occurs at a lower than expected frequency throughout most of the human genome but at a higher than expected frequency in small portions of DNA that are referred to as CpG islands. These CpG islands are often concentrated near gene transcription start sites, the promoter regions where the transcription of DNA to RNA begins. In the normal cell, most of the CpG dinucleotides at gene promoter regions are unmethylated, whereas CpG islands found at other portions of the genome are generally methylated. The absence of CpG island methylation is a hallmark of an active transcription site that is capable of transcribing DNA to RNA. In cancer cells, this pattern of CpG methylation becomes disrupted: CpG islands in promoter regions of selected genes have an unusually high likelihood of methylation, but CpG dinucleotides that fall outside of promoter regions are less likely than normal to be methylated (Herman and Baylin 2003).The methylation of CpG islands, in association with chromatin modifications that accompany the change, prevents the transcription of the gene's DNA, resulting in transcriptional silencing of the gene. Transcriptional silencing of genes that normally possess antitumor activity results in abnormal cellular events, which contribute to tumor progression. Thus, epigenetic gene silencing is a second mechanism, in addition to gene mutation, by which the production of tumor suppressing genes is disrupted (Jones and Baylin 2002).CpG islands, frequently located at the 5'-end regulatory regions of genes, are subject to epigenetic modifications including DNA methylation and histone modification that are known to play an important role in regulating gene expression (Jones and Baylin 2002).In normal cells, the majority of promoter CpG islands are protected from this epigenetic event and thus they are unmethylated. Conversely, in cancer cells, several promoter CpG islands are hypermethylated

and form a closed repressive chromatin configuration that affects the transcription initiation of the corresponding genes (Esteller *et al.,* 2001, 2002; Baylin and Ohm 2006).The term epigenetic refers to information which is transmitted from the parental genome to the next generation of cells which is not encoded by the primary DNA sequence. Epigenetic mechanisms are essential for the regulation of gene expression and genome integrity in normal cells (Bird, 2002; Li, 2002; Jaenisch and Bird 2003).Epigenetic information is often transmitted by methylation of the 5 carbon position of cytosine within a CpG dinucleotide, also referred to as DNA methylation. CpG dinucleotides are under-represented in the genome, but over-represented in short regions of 500– 4,000 bp (base pair) in length, known as CpG islands, which are rich in CpG content (Bird, 2002; Takai and Jones 2002).CpG islands are present in the proximal promoter regions of about 60% of the genes in the mammalian genome and are, generally, unmethylated in normal cells. Patterns of DNA methylation and chromatin structure are profoundly altered in neoplasia and include genome wide losses of and regional gains in DNA methylation (Feinberg and Vogelstein 1983).Global hypomethylation was shown to cause genomic instability which in turn may promote secondary genetic alterations (Eden *et al*., 2003; Gaudet *et al*., 2003), whereas local hypermethylation of promoter regions is associated with transcriptional silencing and can lead to the loss of tumor-suppressor gene function. The molecular mechanisms of epigenetic silencing during tumor formation are only partially understood. In normal cells the pattern of CpG methylation is brought about by a group of enzymes known as the DNA methyltransferases (DNMT). The DNMTs known to date are DNMT1, DNMT1b, DNMT1o, DNMT1p, DNMT2, DNMT3A, and DNMT3b with its isoforms and DNMT3L (Robertson, 2002). Methylation can be de novo (when CpG dinucleotides on both DNA strands are unmethylated) or maintenance (when CpG dinucleotides on one strand are methylated). DNMT1 has de novo as well as maintenance methyltransferase activity, and DNMT3A and DNMT3b are powerful de novo methyltransferases (Marzo *et al*., 1999; Robertson *et al.,* 2000; Costello and Plass 2001)(Figure 5).Methylation of various biological molecules including DNA is dependent on S-adenosylmethionine (SAM), the principal biological methyl group donor (Stern *et al.,* 2000; Yi *et al.,* 2000). Considering the critical role of methylation in various cellular processes, it is understandable that any alteration in the availability of SAM may have a profound effect on cellular growth, differentiation and function in both health and illness. Methylenetetrahydrofolate reductase (MTHFR), a rate-limiting enzyme in the conversion of homocysteine to methionine, which is the substrate for

SAM, may play a regulatory role in the folate metabolism and methylation process (Bolander, 2002). Elevated homocysteine levels, either due to MTHFR mutation or a diet deficient in cofactors for the methylation cycle, result in the formation of S-adenosyl L-homocysteine, which is a competitive inhibitor of SAM. This, as a result, affects methylation in various biological processes.

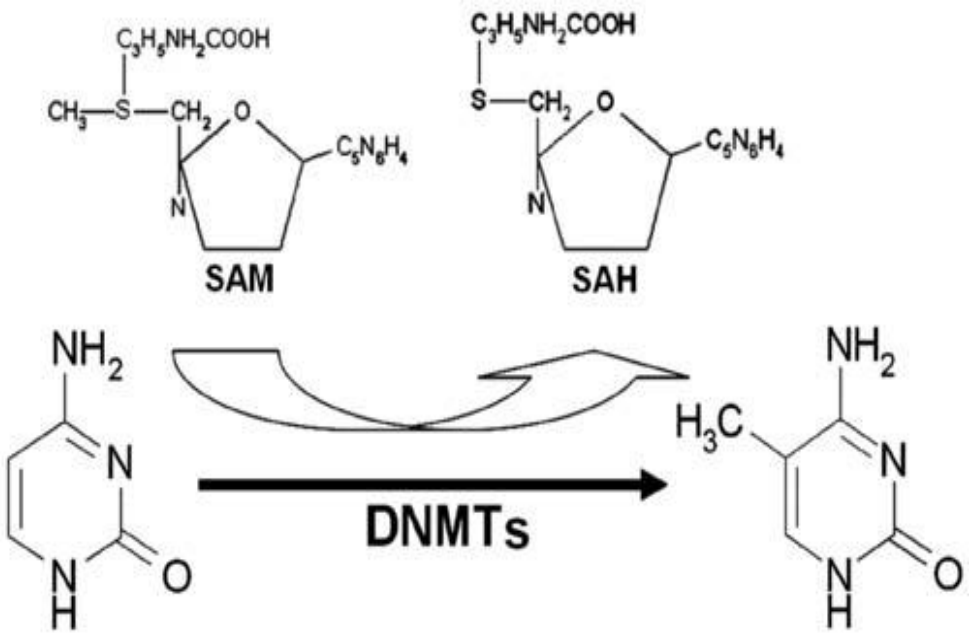

Figure 5: Cytosine gets converted into 5'methyl cytosine by the action of S-adenosylmethionine (SAM) which donates methyl groups and is converted to S-adenosyl homocysteine (SAH) in presence of DNMTs

(Source: Review article on role of methylation)

Epigenetic modifications include DNA methylation and covalent modification of histones. These alterations are reversible but very stable and exert a significant impact on the regulation of gene expression and the development of vertebrates (Santos and Dean 2004). In mammals, m^5C is primarily located in CpG islands of promoter and first exon sequences, which exhibit highly conservative DNA methylation pattern. CpG islands are 0.5 to several kb DNA sequences that contain 60-70% of CpG dinucleotides (Baylin and Herman 2000; Robertson and Jones 2000; Momparler, 2003; Cottrell, 2004). Human genome contains 29000 CpG islands (Clark and Melki 2002) and approximately half of genes possess these islands (Attwood *et al.,* 2002; Das and Singal 2004). Completely methylated CpG islands are found only in promoters of un-transcribed autosomal genes and transcriptionally silenced genes of inactive female X-chromosomes (Baylin and Herman 2000). The N-terminal tails of histones are epigenetically modified by histone acetyltransferases (HATs), histone deacetylases (HDACs) and histone methyltransferases (HMTs). These enzymes acetylate, deacetylate or methylate ε and guanidine amine groups of histone Lys (K) or Arg (R) amino acid residues, respectively. The multiple covalent modifications on the same histone tail create specific epigenetic patterns that switch genes between their active and transcriptionally inactive stages and correlate with distinct biological events (Zhang and Reinberg 2001; Moggs *et al.,* 2004). HDACs, HATs, HMTs and DNMTs play crucial roles in the epigenetic regulation of gene expression involved in carcinogenesis (Sun *et al.,* 1997; Geiman *et al.,* 2004). Alteration of transcriptionally active euchromatin to transcriptionally inactive heterochromatin requires histone remodelling enzymes HDACs, HATs, HMTs (Suv39h1/2, G9a, EZH2) and ATP-dependent chromatin remodeling enzymes (*e.g.* hSNF2H). Methylation level of K4, K9 and K27 amino acid residues of H3 histone corresponds to euchromatin, facultative heterochromatin and constitutive heterochromatin structures, respectively (Geiman *et al.,* 2004). HMT (Suv39h1) attaches methyl group to K9 amino acid residues of H3 histone that further recruits heterochromatin protein 1 (HP1). Interactions of HP1 with methylated K9 of H3 histone and transcriptional complex components are essential for formation and maintenance of heterochromatin structure (Bannister *et al.,* 2001; Smothers and Henikoff 2001; Peters *et al.,* 2002; Cammas *et al.,* 2004; Verschure *et al.,* 2005).CpG islands, frequently located at the 5′-end regulatory regions of genes, are subject to epigenetic modifications including DNA methylation and histone modification that are known to play an important role in regulating gene expression (Jones and Baylin 2002). In normal cells, the majority of promoter CpG islands are protected from this epigenetic event and thus they are

unmethylated. Conversely, in cancer cells, several promoter CpG islands are hypermethylated and form a closed repressive chromatin configuration that affects the transcription initiation of the corresponding genes (Esteller *et al.*, 2001, 2002; Baylin and Ohm 2006).

DNA hypermethylation inhibits gene transcription: Two mechanisms have been proposed to account for transcriptional repression via DNA methylation. In the first mechanism, DNA methylation directly inhibits the binding of transcription factors (TFs) such as AP-2, c-Myc/Myn, E2F and NFkB to their binding sites within promoter sequence. In this mechanism, CpG dinucleotides have to be present within the binding site of TFs, which are sensitive to methylation of CpG dinucleotides (Tate and Bird 1993; Singal and Ginder 1999)(Figure 6b).The second mode of repression includes a binding of proteins specific for m5CpG dinucleotides to methylated DNA. Methylated DNA recruits m5CpG-binding (MeCP) and m5CpG-binding domain (MBD) proteins. MeCP1 and MeCP2 bind specifically to methylated DNA in whole genome and form spatial obstacle that unable binding of TFs to promoter sequences (Figure 2). MeCP1 represses transcription of specific genes, which are controlled by densely methylated promoters containing more than ten m5CpG dinucleotides. MeCP2 can bind to a single symmetrically located m5CpG pair in two DNA strands (Hendrich and Bird 1998) (Figure 6c). MBD protein family includes MBD1, MBD2, MBD3 MBD4, and uncharacterized Kaiso complex, which binds to methylated DNA. MBD1 binds to symmetrically methylated CpG dinucleotides and inhibits gene expression by blocking TFs interaction with the promoter (Fujita *et al.*, 2000). MBD2 may bind to methylated DNA and actively demethylates DNA *in vivo* and *in vitro* (Ng HH *et al*., 1999; Szyf *et al*., 2004). MBD3 is targeted to methylated DNA through association with the MBD2 and is a component of the chromatin remodeling protein complex (Ballestar *et al*., 2003). MBD4 is thymine and uracil glycosylase involved in DNA mismatch repair, formed during C and m5C deamination, respectively (Hendrich *et al.*, 1999; Fujita *et al.*, 2003). MBD1, MBD2, MeCP2, and Kaiso complex are able to interact with HDAC1 and HDAC2, which deacetylate histones and remodel chromatin structure (Baylin and Herman 2000; Robertson and Jones 2000; Brown and Strathdee 2002; Das and Singal 2004; Laird, 2005).

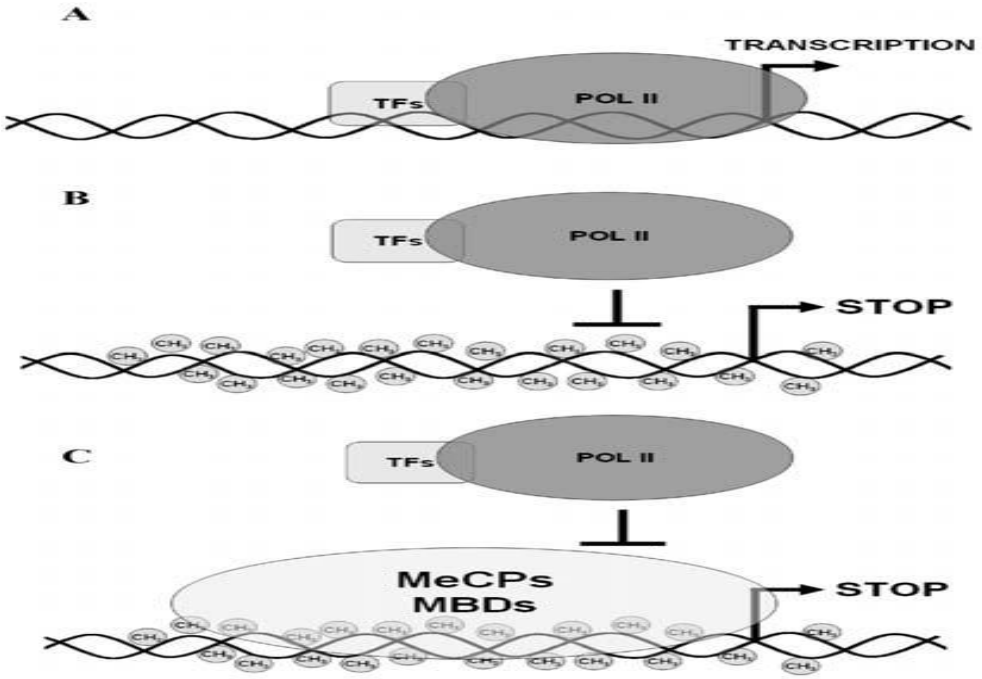

Figure 6: Repression of transcription via CpG dinucleotide methylation. Promoter sequence binds transcription factors (TFs) and RNA polymerase II (POL II) that initiates transcription (A). Methylation of CpG within promoter binding site directly inhibits requirement of TFs and represses transcription (B). Methylated DNA binds m5CpG binding (MeCPs) and m5CpG-binding domain (MBDs) proteins forming spatial obstacle that prevents binding of TFs to promoter sequence

(Source: Review article on role of methylation)

DNA methylation in cancer has become the topic of intense investigation. As compared with normal cells, the malignant cells show major disruptions in their DNA methylation patterns (Baylin and Herman 2000).Hypermethylation involves CpG islands whereas Hypomethylation usually involves repeated DNA sequences, such as long interspersed nuclear elements (Ehrlich, 2002).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 (Clark and Melki 2002).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 ($p16^{INK4a}$, $p15^{INK4a}$, *Rb*, p^{14ARF}) genes associated with DNA repair (*hMLH1*, *BRCA1, MGMT*), apoptosis (*DAPK, TMS1*), angiogenesis (*THBS1, VHL*), invasion (*CDH1*, *TIMP3*), drug resistance, detoxification, differentiation, and metastasis (Jones and Baylin 2002).

Hypomethylation is the second kind of methylation defect that is observed in a wide variety of malignancies (Feinberg and Vogelstein 1983; Kim *et al*., 1994). It is common in solid tumors such as metastatic hepatocellular cancer (Lin *et al.*, 2001), in cervical cancer (Lin *et al.*, 2001), prostate tumors (Bedford and Helden 1987), and also in hematologic malignancies such as B-cell chronic lymphocytic leukemia (Ehrlich 2002). The global hypomethylation seen in a number of cancers, such as breast, cervical, and brain, show a progressive increase with the grade of malignancy (Ehrlich 2002). The pericentric heterochromatin regions on chromosomes 1 and 16 are heavily hypomethylated in patients with immunodeficiency, centromeric instability, and facial abnormalities and in many cancers. A mutation of *DNMT3b* has been found in patients with immunodeficiency, centromeric instability, and facial abnormalities, which causes the instability of the chromatin (Hansen *et al*., 1999; Okano *et al*., 1999). Hypomethylation has been hypothesized to contribute to oncogenesis by activation of oncogenes such as *cMYC* and *H-RAS* (Feinberg and Vogelstein 1983) or by activation of latent retro transposons (Singer *et al.*, 1993; Alves *et al*., 1996) or by chromosome instability (Tuck-Muller *et al*., 2000). Long interspersed nuclear elements are the most plentiful mobile DNAs or retro transposons in the human genome. Hypomethylation of these mobile DNAs causes transcriptional activation and has been found in many types of cancer, such as urinary bladder cancer (Jurgens *et al*., 1996).

2.4 Literature about *hMLH1* Gene

The official name of this gene is "mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)." *hMLH1* is the gene's official symbol. The *hMLH1* gene is also known by other names, as COCA2, DNA mismatch repair protein *hMLH1*, FCC2, HNPCC, mutL (E. coli) homolog 1 (colon cancer, nonpolyposis type 2) and MutL protein homolog 1. The *hMLH1* gene is located on the short (p) arm of chromosome 3 at position 21.3 from base pair 37,034,840 to base pair 37,092,336 (Figure 7). About 50 percent of all cases of Lynch syndrome with an identified gene mutation are associated with mutations in the *hMLH1* gene. Several hundred *hMLH1* mutations that predispose people to colorectal cancer and other cancers have been found. These mutations prevent the production of *hMLH1* protein or lead to an altered version of this protein that does not function properly. When the *hMLH1* protein is absent or ineffective, the number of mistakes that are left unrepaired during cell division increases substantially. If the cells continue to divide, errors accumulate in DNA; the cells become unable to function properly and may form a tumor in the colon or another part of the body. The DNA mismatch repair (MMR) protein *hMLH1* is encoded by the MutL homolog 1 (*hMLH1*) gene in humans and is a homologue of the DNA MMR gene mutL of Escherichia coli. The MMR function is associated with DNA replication, to correct for deficiencies in DNA polymerase proofreading function. A missing gene or mutations of this gene and other MMR genes (MSH2, MSH6, or PMS2) leads to microsatellite instability (MSI) and this dysfunction is highly associated with hereditary non-polyposis colon cancer (HNPCC or Lynch syndrome) (Bronner *et al.*, 1994). The *hMLH1* gene provides instructions for making a protein that plays an essential role in DNA repair. This protein fixes mistakes that are made when DNA is copied (DNA replication) in preparation for cell division. The *hMLH1* protein joins with another protein, the PMS2 protein, to form an active protein complex. This protein complex coordinates the activities of other proteins that repair mistakes made during DNA replication. The repairs are made by removing a section of DNA that contains mistakes and replacing the section with a corrected DNA sequence (Jascur and Boland 2006). Human MutL homologue or *hMLH1* is a member of the mismatch repair system whose function is to replicate the genome faithfully (Ahuja *et al.*, 1998). Deficiencies in this system result in mutation rates 100-fold higher than those observed in normal cells (Thomas *et al.*, 1996; Jiricny, 2006).

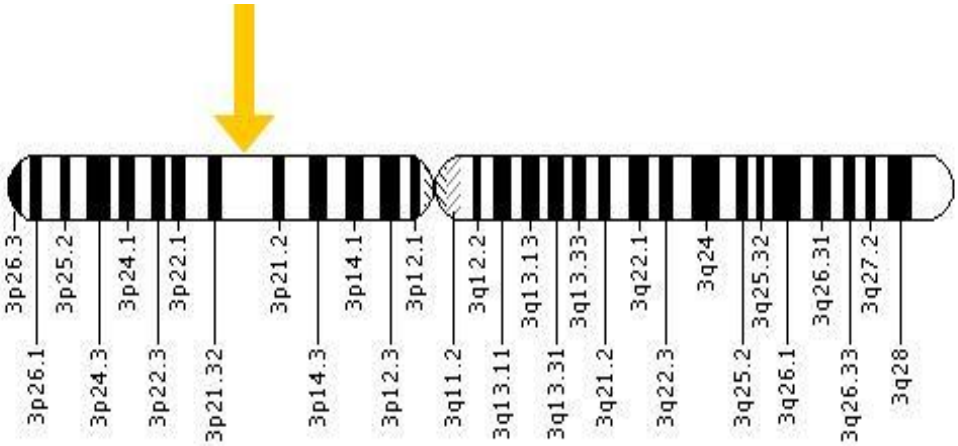

Figure 7: *hMLH1* gene on short arm of chromosome 3 at position 21.3

(Source: Handbook Genetics home reference)

A variety of genetic lesions has been demonstrated to be involved in esophageal cancer, including p53-Rb pathway with gene amplifications, loss of heterozygosity or homozygous deletions, point mutations, and chromosomal rearrangements (Chen and Yang 2001; Wang *et al.,* 2002; Enzinger and Mayer 2003). DNA methylation of the promoter region of certain cancer-associated genes is one potential early detection biomarker (Kawakami *et al.,* 2000; Hibi *et al.,* 2001; Yao *et al*., 2005). Previous studies have shown that smoking and alcohol consumption increase the risk of developing microsatellite-unstable tumors (Slattery *et al.,* 2000; Slattery *et al.,* 2001). The exact mechanism of DNA hypermethylation by alcohol is unknown. However, it has been hypothesized that alcohol could influence carcinogenesis by influencing mucosal cell proliferation and related histological changes (Kune and Vitetta 1992). These changes have been associated with mucosal hyper regeneration, which may make the mucosa more susceptible to the action of other carcinogens such as cigarette smoke (Kune and Vitetta 1992). Therefore, alcohol consumption might increase the bioavailability of DNA-binding smoke components in the mucosa of the upper digestive tract, increasing the plasma levels of these compounds, or might modify the metabolism of pro-carcinogenic compounds by inducing specific metabolic pathways involving an aberrant mismatch repair system (Izzotti *et al.,* 1998). The DNA MMR protein *hMLH1* is encoded by the MutL homolog 1 (*hMLH1*) gene in humans and is a homologue of the DNA MMR gene *mutL* of *Escherichia coli*. The MMR function is associated with DNA replication, to correct for deficiencies in DNA polymerase proofreading function. A missing gene or mutations of this gene and other MMR genes (*MSH2*, *MSH6*, or *PMS2*) leads to microsatellite instability (MSI) and this dysfunction is highly associated with hereditary non-polyposis colon cancer (HNPCC or Lynch syndrome) (Bronner *et al*., 1994).Loss of function of the DNA mismatch repair gene *hMLH1* by hypermethylation of its promoter has been described in different cancer types, such as colorectal and endometrial cancers (Herman *et al.,* 1999; Esteller *et al.,* 2001). Methylation of the *hMLH1* gene in 3p21.3 and its correlation with a mismatch repair defect and high microsatellite instability (MSIH) is well characterised in sporadic colorectal cancer, where this phenotype is associated with better patient survival (Sinicrope *et al*., 2006).

It has been shown that methylation in the promoter region of *hMLH1* correlates with decreased activity of the gene (Kane *et al*., 1997). Next to the main cancer type where this gene is inactivated, HNPCC, this gene is epigenetically inactivated also in other types of cancer, for example, in sporadic endometrial carcinoma (Esteller *et al*., 1998), gastric cancers

(Fleisher *et al*., 1999), sporadic CRC (Kane *et al.,* 1997; Herman *et al*., 1998), ovarian tumors (Gras *et al*., 2001a), NSCLC (Wang *et al*., 2003), oral squamous cell carcinoma (SCC) (Czerninski *et al*., 2009), neck SCC (Liu *et al*., 2002; Steinmann *et al*., 2009), and acute myeloid leukemia (AML) (Seedhouse *et al*., 2003). Constitutional methylation of the *hMLH1* gene, characterized by soma-wide methylation of a single allele and transcriptional silencing, has been identified in a subset of Lynch syndrome cases lacking a sequence mutation in *hMLH1* (Gazzoli *et al*., 2002; Suter *et al*., 2004; Hitchins *et al*., 2007). This particular example provides strong support for the proposal that methylation of a DNA repair gene can be a crucial mechanism in carcinogenesis.In tumor types that rarely show the classic MMR-deficient phenotype and that are rare in HNPCC kindreds, such as breast and lung carcinomas or gliomas, *hMLH1* is not hypermethylated (Esteller *et al.,* 1998). However, *hMLH1* promoter hypermethylation occurs in the majority of sporadic colorectal, endometrial, and gastric carcinomas showing MSI (Ottini *et al.,* 1997; Yamamoto *et al.,* 1997).

Cancer and some chronic inflammatory conditions have been associated with genomic instability (Ishitsuka *et al.,* 2001; Gao *et al.,* 2005). The MMR system maintains genomic integrity, and it is accepted that defects in MMR genes are responsible for the microsatellite instability (MSI) observed in different diseases (Brentnall *et al.,* 1995; Lynch and Hoops 2002). Approximately 27% of esophageal tumors deficient in *hMLH1* repair gene expression have been associated with MSI (Hayashi *et al.,* 2003). It was also observed *hMLH1* hypermethylation to be associated with MSI in about 42% of esophageal cancers (Vasavi and Hasan).

MMR gene silencing has been reported in several cancers and inflammatory conditions such as inflammatory bowel disease, head and neck cancers, and cancers of the GI tract (Bubb *et al.,* 1996; Wang *et al.,* 2001; Kang *et al.,* 2002). In most of these studies, immunohistochemistry and mRNA expression analysis showed the absence of *hMLH1* protein; however, no mutations were found to be responsible for this (Liu *et al.,* 1995; Kane *et al.,* 1997). Epigenetic silencing through promoter methylation of the *hMLH1* repair gene was associated with loss of *hMLH1* protein expression (Razin *et al.,* 1980; Kane *et al.,* 1997). In esophageal cancers, there are controversial reports regarding the association of hypermethylation with *hMLH1* gene silencing (Nie *et al.,* 2002; Hayashi *et al.,* 2003).

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. For that First of all Genomic DNA was Isolated from esophageal samples and then the DNA sample was treated with sodium bisulfite solution which converts by deamination unmethylated cytosine's present in DNA into uracil and does not affect methylated cytosines, due to this methylated and unmethylated cytosines can be distinguished. The mechanism behind the bisulfite treatment is shown in Figure 8. Then methylation specific polymerase chain reaction (MS-PCR) was performed. The principle of the method lies in the amplification of the target DNA (already bisulfite treated) by different primer sequence;

- one for methylated version of the gene
- one for the unmethylated version of the gene

Thus by visualising the MS-PCR product we can easily determine whether amplification is by methylated or unmethylated primers.

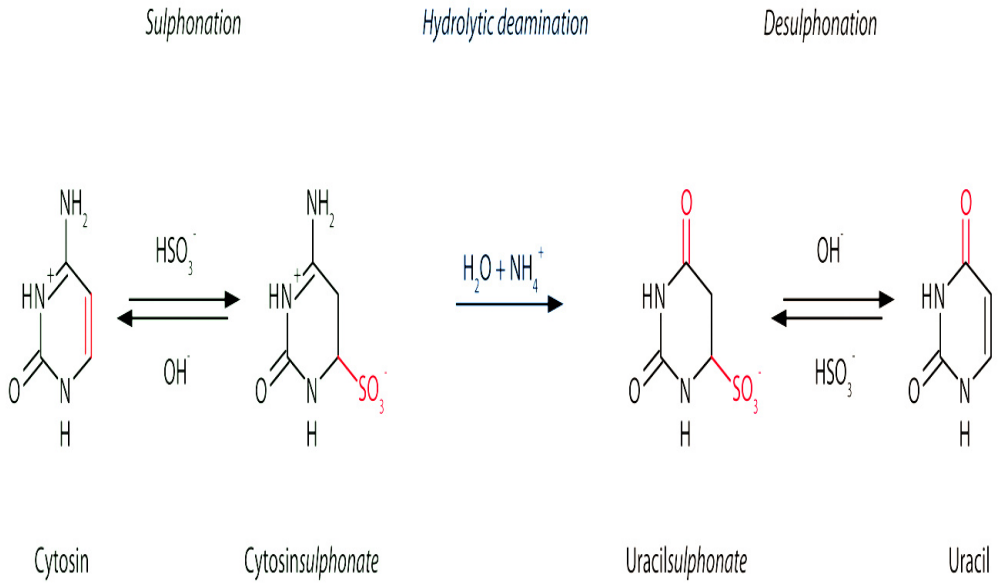

Figure 8: Conversion of cytosine to uracil by bisulfite treatment

(Source: Bisulfite sequencing Wikipedia)

MATERIALS AND METHOD:

3.1 Case Control Study

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 (*hMLH1*) with esophageal cancer. Also further an association of hypermethylation of gene with esophageal cancer in relation to clinicopathological features of Gender and Age was evaluated and thus further two sub-groups of Gender as Male and Female and Five more sub-groups of age as upto-39 years, 40-49 years, 50-59 years, 60-69 years, and above-70 years were designed and are listed in Table 2 in results. The Esophageal cancer samples were collected from Endoscopic section of Shri Maharaja Hari Singh (S.M.H.S) Hospital including resected esophageal samples from Department of Surgery, S.M.H.S Hospital Srinagar. The controls were taken from the adjacent tissue of the Esophageal cancer.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. No patient received pre-operative radiation or chemotherapy (Coia *et al*., 1995).Record was maintained of the complete case history of the patients.

The various methods that were used to analyse the epigenetic silencing of DNA mismatch repair gene by its promoter hypermethylation at CpG islands as a part of this study are described under:

3.2 Cases (Patients and Specimens)

All the esophageal cancer patients that were referred to endoscopy section Government Shri Maharaja Hari singh (S.M.H.S) Hospital and operated in the Department of Surgery, Government S.M.H.S Hospital, Srinagar during the study period were included in the study irrespective of their age, gender and stage of the cancer.

3.2a. Inclusion Criteria

The diagnosis of esophageal cancer was based on the standard histopathological criteria. The criteria for including a subject as case in the study were:

- All histopathologically confirmed patients irrespective of Cancer stage, age, and gender.

- Native patients belonging to Kashmir valley.

3.2b. Exclusion Criteria

Under the following conditions the patients were not recruited in the study:

- Patients who had received prior chemo or radiotherapy.
- Patients not belonging to Kashmir valley.
- Patients who suffered from any other kind of malignancy

3.3 Controls (Adjacent Normals)

Esophageal biopsies from Endoscopic section of S.M.H.S Hospital including resected esophageal samples from Department of Surgery, S.M.H.S Hospital which were histopathologically confirmed as normal were taken as controls.

3.3a. Inclusion criteria

- All histopathologically confirmed Normal Esophageal tissues.
- Native population of the Kashmiri origin.

3.3b. Exclusion criteria

- Patients who suffered from any other kind of malignancy.
- Patients not of Kashmiri origin.

3.4 Collection of Esophageal Tissue Samples

The Esophageal cancer and adjacent control sample were collected from the Endoscopic Section and Department of Surgery of Government S.M.H.S Hospital and were put in sterilized labelled plastic vials (50 ml volume) containing 10 ml of normal saline and transported from the theatres to the laboratory on ice and stored at -80 ^{o} C for further analysis.

Esophageal Tissue samples were divided into two parts:

- One part was sent to histopathological diagnosis.
- Other half was stored at -80^{0}C 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. Complete Clinicopathological record was maintained for both cases and controls.

3.5 Genetic Analysis of Histopathologically Confirmed Cases and Controls

3.5a. Extraction of Genomic DNA

High Molecular Genomic DNA from the histopathologically confirmed fresh Esophageal Cases and adjacent control tissue samples was extracted by kit based method. The kit used was Quick- g DNA^{TM} MiniPrep supplied by ZYMO RESEARCH. The protocol followed was as directed by the company as:

- Briefly, 25 to 50 mg of tissue was taken and the cells were mechanically homogenised in 500 µl Genomic Lysis Buffer (provided in the kit).
- The lysate was centrifuged at 10,000 X g for 5 min. and the supernatant transferred to Zymo-Spin^{TM} Column in a Collection Tube and again centrifuged at 10,000 X g for 1 min.
- Flow through along with the Collection tube was discarded and the Zymo-Spin^{TM} Column transferred to a new Collection tube and 200 µl of DNA Pre-Wash Buffer (provided in the kit) was added to the tube and centrifuged at 10,000 X g for 1 min., followed by the addition of 500 µl of g- DNA Wash Buffer to the Zymo-Spin^{TM} Column and again centrifuged at 10,000 X g for 1 min.
- The spin column was transferred to a clean micro centrifuge tube and DNA was eluted from it into the micro centrifuge tube by addition of 50 µl of DNA Elution Buffer (provided in the kit).

The DNA eluted was stored at 4^0C for a short time but the vials were kept at -20^oC for longer duration storage for further investigation.

3.5b. Qualitative Analysis of Genomic DNA

The integrity of the genomic DNA was examined by gel electrophoresis using 1 % agarose gel to which 10 µl/ 50ml (of gel solution) of fluorescent dye ethidium bromide was added during its cooling and then gel was cast and 20 µl wells were cast into it by usage of suitable combs. 2µl of each DNA sample was mixed with 1µl of 1X DNA loading dye and

was loaded in the gel. Electric current was applied at 50 volt until DNA entered in to the gel and was raised to 70 volt for rest of the run. Run was stopped when the dye had travelled nearly 2/3rd of the gel. DNA in the gel was visualized with the help of Gel doc system (AlphaimagerTM 2200, Alpha Innotech Corporation) under UV light and picture was captured by using CCD camera system and is shown in Figure 9.

1X loading dye consists of:

- 4.16 mg bromophenol blue
- 4.16 mg xylene cyanol
- 0.66g sucrose
- Final volume 1ml with deionized water

3.5c. Quantitative Analysis of Genomic DNA

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) and the concentration was determined by using the fact that absorbance 1 absorbance unit equates to 50 µg/ cm^3 and therefore, the concentration of DNA sample is given by the following equation:

DNA (µg/ml) = A_{260} x 50 x dilution factor

The ratio of 260/280nm was calculated and the DNA samples for which the ratio was 1.7-1.9 was considered for the future use. The DNA was stored at 4^oC for a short time but the vials were kept at -20^oC for longer duration storage.

3.5d. Bisulfite Treatment to Genomic DNA

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*).*The above extracted Genomic DNA was treated with sodium bisulphite solution under carefully controlled conditions by which unmethylated cytosine's 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. The protocol is as under:

- 500-1000 ng of above isolated DNA samples were taken in different eppendorf tubes.

- And to each tube 5 µl of M-Dilution Buffer was added and final volume was made to 50 µl with distilled water.
- Then 100 µl CT-Conversion Reagent was added to each tube (preparation of CT-Conversion reagent is given below) and all tubes were placed in dark at 50^0-55^0c for 12-16 hrs.
- After that all samples were placed on ice for 5-10 mins.
- 400 µl of M-binding buffer was added into the Zymo-Spin™ IC Column and column was placed into a provided Collection Tube.
- Sample was loaded (from step 4) into the Zymo-Spin™ IC Column containing M-binding buffer. Cap was closed and column was mixed by inverting several times.
- Centrifugation was done at full speed (≥10,000× *g*) for 30 seconds. Flow-through was discarded.
- Now 100 µl of M-Wash Buffer was added to the column and Centrifugation was done at full speed for 30 seconds.
- 200 µl of M-Desulphonation Buffer was added to the column and was incubated at room temperature (20^0C-30^0C) for 15-20 minutes. After the incubation, centrifugation was done at full speed for 30 seconds.
- Again 200 µl of M-Wash Buffer was added to the column and was centrifuged at full speed for 30 seconds; another 200 µl of M-Wash Buffer was added and again centrifuged for an additional 30 seconds.
- Column was placed into a 1.5 ml micro centrifuge tube and 15 µl of M-Elution Buffer was added directly to the column matrix. Centrifugation for 30 seconds at full speed was done to elute the DNA.

The DNA was ready for immediate analysis or can be stored at or below -20^0C for later use.

Preparation of CT-Conversion Reagent

The CT-Conversion Reagent supplied within this kit was a solid mixture and must be prepared prior to first use. Prepare as follows:

- 790 µl of M-Solubilisation Buffer and 300 µl of M-Dilution Buffer were added to the tube of CT Conversion Reagent.

- Mixing was done at room temperature with frequent vortexing or shaking for 10 minutes.
- 160 µl of M-Reaction Buffer was added and mixed for an additional 1 minute.

3.5e. 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. This alternative method of methylation analysis also uses bisulfite-treated DNA but avoids the need to sequence the area of interest (Herman *et al., 1996*). Instead, primer pairs were designed themselves to be "methylated-specific" by including sequences complementing only unconverted 5-methylcytosines, or, on the converse, "unmethylated-specific", complementing thymines converted from unmethylated cytosines. Methylation was determined by the ability of the specific primer to achieve amplification. This method was particularly useful to interrogate CpG islands with possibly high methylation density, as increased numbers of CpG pairs in the primer increase the specificity of the assay. Placing the CpG pair at the 3'-end of the primer also improves the sensitivity. The initial report using MSP described sufficient sensitivity to detect methylation of 0.1% of alleles. In general, MS-PCR and its related protocols were considered to be the most sensitive when interrogating the methylation status at a specific locus. The principle method lies in the amplification of the target DNA (already Bisulfite treated) by different primer sequence:

- One for Methylated version of the gene
- One for the Unmethylated version of the gene

Thus by visualising the PCR product we have easily determined whether amplification was by Methylated or Unmethylated primers. This determines whether CpG's were Methylated or Unmethylated.

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 and are shown in the Table 1 (Steven *et al.,* 1999, 2001; Hong *et al.,* 2005; Edward *et al.,* 2006).

Table 1: Primer Sequences and annealing Temperatures of *hMLH1* gene

Gene	Sequence (5′-3′)		*Tm* (◦C)	Size (bp)	PCR cycles
(U)	F	5'-TTTTGATGTAGATGTTTTATTAGGGTTGT-3'	60	115	30
	R	5'-ACCACCTCATCATAACTACCCACA-3'			
(M)	F	5'-ACGTAGACGTTTTATTAGGGTCGC-3'	58	110	30
	R	5'-CCTCATCGTAACTACCCGCG-3'			

For methylation-specific polymerase chain reaction (MS-PCR) the total reaction volume was 25 µl containing:

- 1.25 µl bisulfite modified DNA (50–100 ng)
- 5 µl of each forward and reverse primer (150 ng of each)
- 1.50 µl of dNTP's (1.25 mM/L)
- 0.2 µl Taq Polymerase (1 U/reaction)
- 2.5 µl 1X PCR buffer (Bangalore Genei, Bangalore) and Final volume was made to
- 25 µl with de-ionized water.

The modified DNA was taken into two PCR vials in equal quantity and same amount of all reagents was added to both the vials except in one vial, methylated primers and in other, non-methylated primers were used. PCR amplification was achieved using a Thermal cycler (Gradient thermal cycler from EPPENDORF MASTERCYCLER PRO).

PCR reactions were started by denaturation at 95^0C for 5 min, followed by 30 cycles at 94^0C for 30 s, 60^0C (for unmethylated *hMLH1*), 58^0C (for methylated *hMLH1*) for 30 s, and 72^0C for 30 s, with a final extension at 72^0C for 4 min.

All MS-PCRs were performed with controls for unmethylated DNA (DNA from normal lymphocytes Negative Control) and methylated DNA [normal lymphocyte DNA treated in vitro with SssI methyltransferase Positive Control (ZYMO RESEARCH)].

3.5f. Electrophoresis of MS-PCR products

After the MS-PCR is over the resulted amplification products was visualized under UV-illumination. 10 µl of each MS-PCR product was directly loaded on 3% agarose, stained with ethidium bromide and visualized under UV illumination.

3.6 Data Analysis And Statistics

The χ^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 and Fishers exact test was used in case of studying the male and female groups as cell frequency was less than 5 in some cells. ORs with 95% CIs 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 92037, USA.

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.

4.1 Methylation Status of Promoter Region of Mis-Match Repair Gene *(hMLH1*) in Esophageal Cancer

To determine the status of Mismatch Repair (*hMLH1) gene* promoter methylation in Esophageal Cancer Cases from the population of Kashmiri origin, Methylation Specific Polymerase chain reaction for *hMLH1* gene was performed in 50 surgically resected histopathologically confirmed esophageal cases and compared with that of 20 histopathologically confirmed normal tissues. The Study included 33 Male cases and 17 Female cases out of which only 04 cases were below the age of 40 years and remaining 46 cases were above the age of 40 years and Controls include 12 Males and 08 Females out of which 02 controls were below the age of 40 years and remaining 18 controls were above the age of 40 years which is tabulated in Table 2.The gel photo of isolated Genomic DNA samples is shown in Figure 9.

Table 2: Number of Males and Females in Cases vs Controls

GENDER	CASES(50)	CONTROLS(20)
Male	**33**	**12**
Female	**17**	**08**
Age (years)		
Upto-39	**04**	**02**
40-49	**06**	**03**
50-59	**13**	**05**
60-69	**20**	**07**
70-above	**07**	**03**

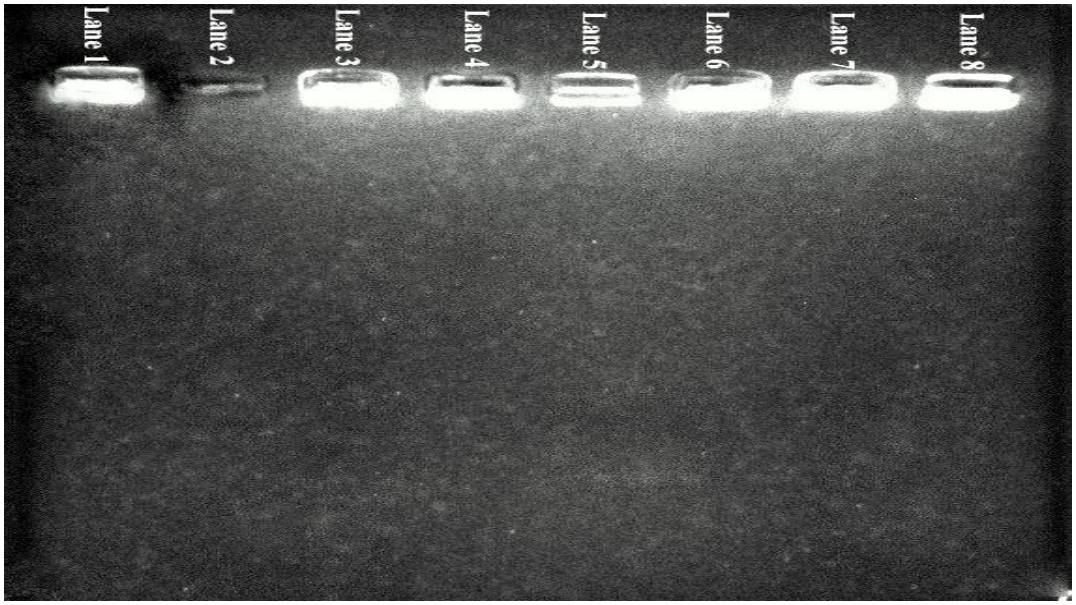

Figure 9: Representative Gel Picture showing the integrity of the Genomic DNA on 1.0% agarose

Lane 1	Gel Image of Genomic DNA 06, histopathologically confirmed Esophageal Cancer Case
Lane 2	No Gel Image of Genomic DNA 01, histopathologically confirmed Esophageal Cancer case
Lane 3	Gel Image of Genomic DNA 17, histopathologically confirmed Esophageal Cancer case
Lane 4	Gel Image of Genomic DNA 18, histopathologically confirmed Esophageal Cancer case
Lane 5	Gel Image of Genomic DNA 67, histopathologically confirmed Esophageal Cancer case
Lane 6	Gel Image of Genomic DNA 68, histopathologically confirmed Esophageal Cancer case
Lane 7	Gel Image of Genomic DNA 24, histopathologically confirmed Esophageal Normal sample
Lane 8	Gel Image of Genomic DNA 31, histopathologically confirmed Esophageal Normal sample

4.2 Result Analysis

Analysis of promoter methylation of *hMLH1* was carried out in resected 50 invasive primary esophageal cancer cases, respectively. Figure 10 and 11 shows representative examples of MS-PCR results. The frequency of promoter hypermethylation was 56% (28 out of 50) for *hMLH1* gene. 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 non-malignant epithelial cells in the primary tumors.

4.2a. Cases vs Controls

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 10: Case 67 and 68). However 22 out of 50 (44%) of the esophageal cancer cases does not showed bands in methylated wells but in unmethylated wells confirms Promoter region of Mismatch Repair Gene (*hMLH1*) is not hypermethylated (Figure 10: Case 05).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 11: Control 18,06 and 08) while in remaining 03 histopathologically confirmed esophageal Controls ,bands were seen in methylated (M) wells as well as in unmethylated wells which confirms that remaining 15% of histopathologically confirmed esophageal Controls were hypermethylated (Figure 11: Control 67,Figure 12: histogram and Table 3). Statistically the Association of promoter Hypermethylation with esophageal cancer was found significant with the p value =0.0028 and was evaluated by χ^2 (Chi square) test with Odds ratio (O.R=7.2121, 95% C.I=1.822-27.79).

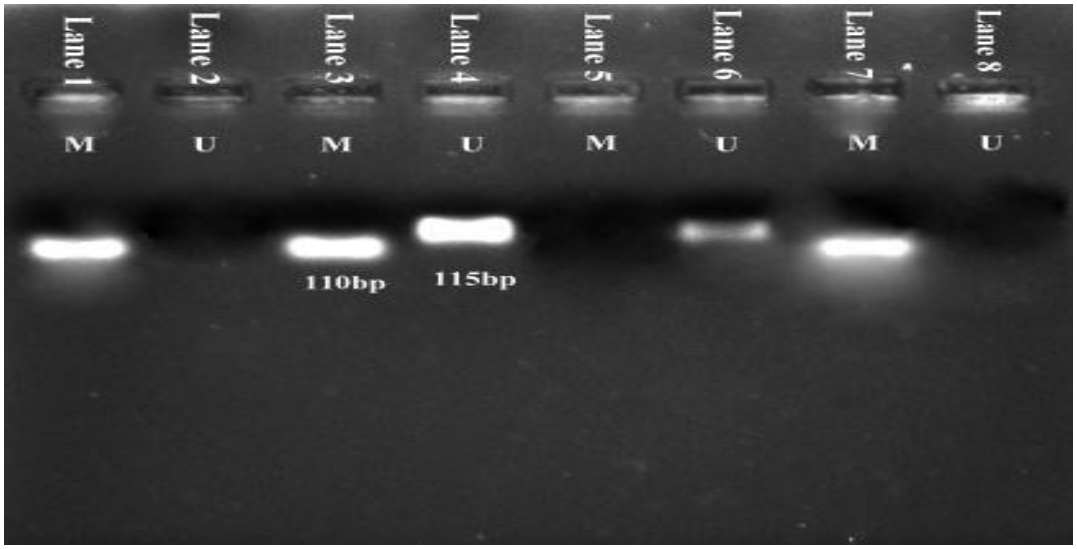

Figure 10: MS-PCR was performed with primers specific for Methylated (**M**) and Unmethylated (**U**) regions of Cases. Product sizes: *hMLH1* Unmethylated, 115 bp; *hMLH1* Methylated, 110 bp

Lane 1	Band Seen in methylated well	Means Esophageal 67 cancer case shows *hMLH1* gene promoter hypermethylation
Lane 2	Band not Seen in unmethylated well	
Lane 3	Band seen in methylated well	Means Esophageal 68 cancer case shows *hMLH1* gene promoter hypermethylation
Lane 4	Band also Seen in unmethylated well	
Lane 5	Band not seen in methylated well	Means Esophageal 05 cancer case does not shows *hMLH1* gene promoter hypermethylation
Lane 6	Band Seen in unmethylated well	
Lane 7	Band seen in methylated well	Means positive control (universal methylated DNA) shows amplification in methylated well only
Lane 8	Band not Seen in unmethylated well	

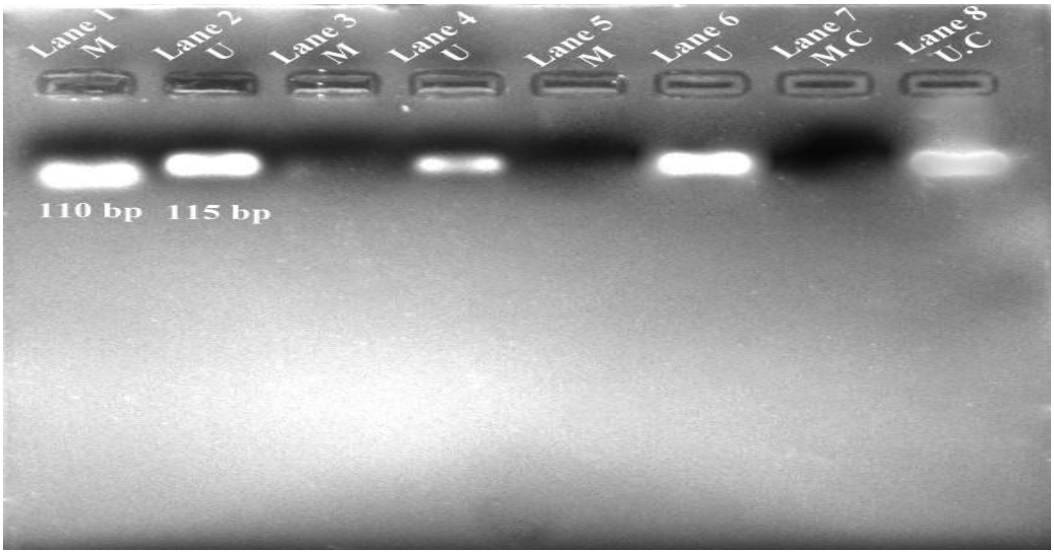

Figure 11: Methylation analysis of MutL homolog 1 (*hMLH1*) gene promoter in Normal Esophageal controls. MSP was performed with primers specific for Methylated (**M**) and Unmethylated (**U**) regions. Product sizes: *hMLH1* Unmethylated, 115 bp; *hMLH1* Methylated, 110 bp

Lane 1	Band Seen in methylated well	Means Normal Esophageal control 67
Lane 2	Band also Seen in unmethylated well	promoter is hypermethylated
Lane 3	Band not seen in methylated well	Means Normal Esophageal control 18
Lane 4	Band Seen in unmethylated well	promoter is not hypermethylated
Lane 5	Band not seen in methylated well	Means Normal Esophageal control 06
Lane 6	Band Seen in unmethylated well	promoter is not hypermethylated
Lane 7	Band not seen in methylated well	Means negative control(lymphocyte
Lane 8	Band Seen in unmethylated well	DNA) shows amplification in unmethylated well only

Table 3: Promoter Region Hypermethylation of Mismatch Repair (*hMLH1*) of Case vs Control as; Total, Hypermethylation, Non-hypermethylation and Frequency

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

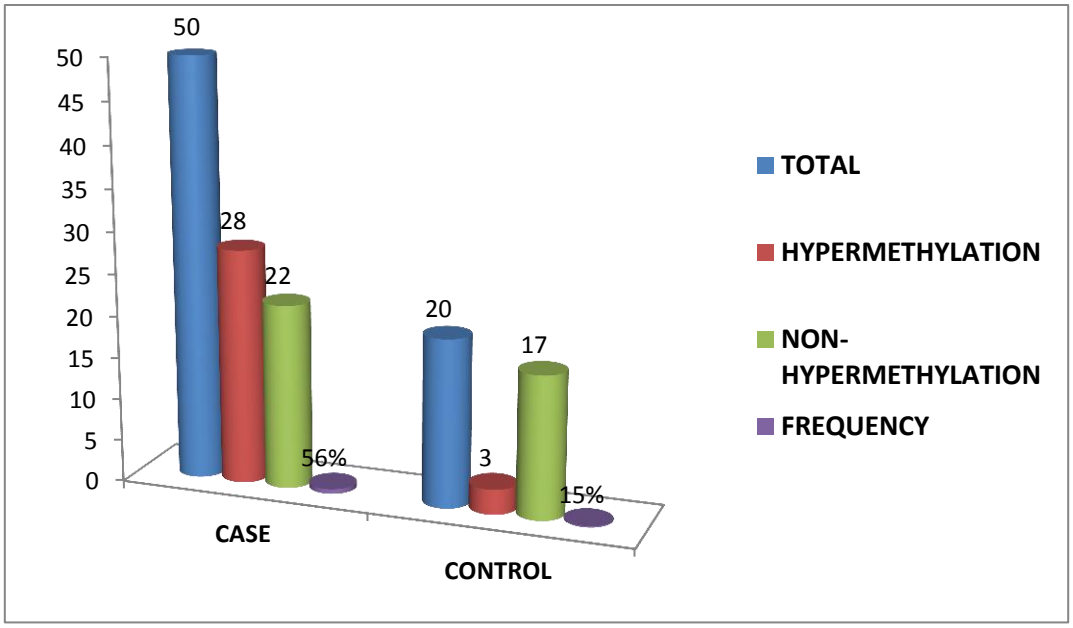

Figure 12: Promoter region hypermethylation of Mismatch Repair (*hMLH1*) gene of Cases vs Controls as; Total, Hypermethylation, Non-hypermethylation and Frequency

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4.3 FURTHER AS REGARDING THE GENDER TWO MORE SUB-GROUPS OF MALE AND FEMALE WERE DESIGNED AND REVEALED THE FOLLOWING DATA

4.3a. Males

Promoter region hypermethylation of *hMLH1* gene was found in 20 Male Cases out 33 (60%) and in Controls 02 out of 12 (16%).which is represented in histogram in Figure 13 and data in Table 4, which is Statistically Significant Association with p=0.0165 and was evaluated by Fishers exact test with odds ratio (O.R=7.6922, 95% C.I=1.446-40.92, p=0.0165)

4.3b. Females

In case of Females Promoter region hypermethylation of *hMLH1* gene was found in 08 Cancer Cases out of 17(47%) and 01 Control out of 08 (12%),which is shown in histogram in Figure 14 and data Tabulated in Table 5.The association was found insignificant statistically because p=0.1822 (O.R=6.2222,95% C.I=0.6225-62.19 and p=0.1822)

4.3c. Total

From the data and results it was found that the frequency of methylation status of MutL homolog 1 *(hMLH1*) gene in Esophageal Cancer Cases was high in Males 20 out of 33 (60%) compared to Females 08 out of 17 (47%) which shows an insignificant association statistically as p=0.3822,odds ratio=1.7310 and 95% C.I=0.5312-5.639. This is represented histogramically in Figure 15 and data Tabulated in Table 6.

Table 4: Promoter Region Hypermethylation of Mismatch Repair gene (*hMLH1*) in Males of Case vs Control as; Total, Hypermethylation, Non-hypermethylation and Frequency

PARAMETER	CASE MALES (33)	CONTROL MALES (12)
HYPERMETHYLATION	**20**	**02**
NON-HYPERMETHYLATION	**13**	**10**
FREQUENCY	**60%**	**16%**

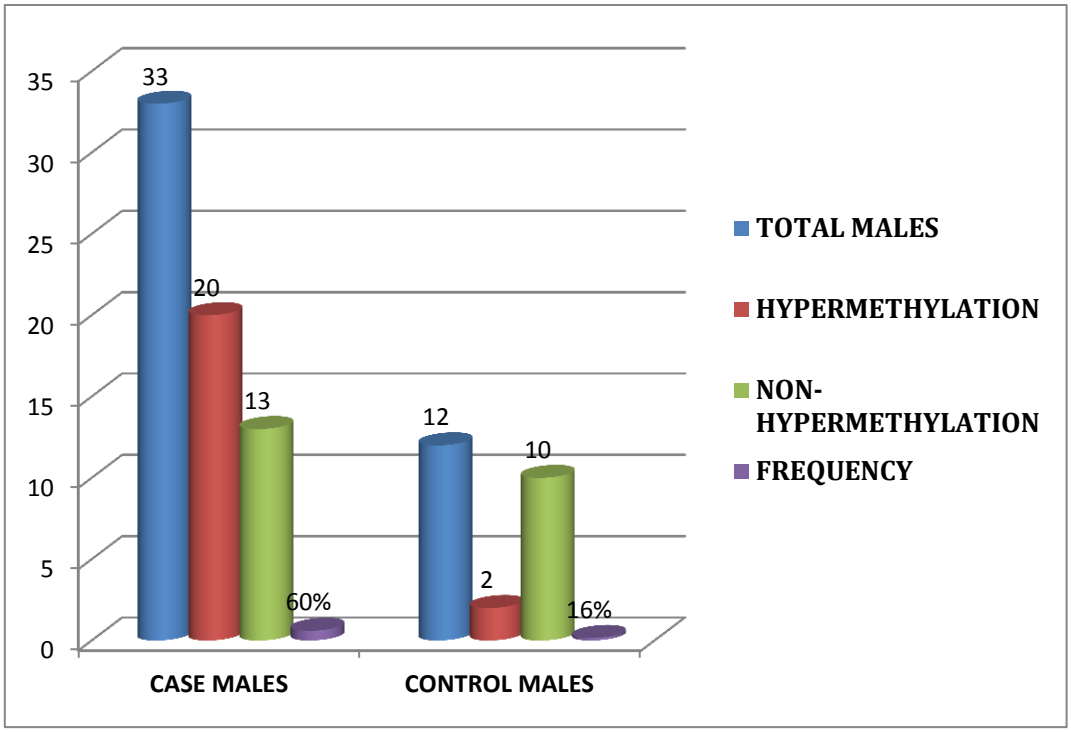

Figure 13: Promoter Region Hypermethylation of Mismatch Repair (*hMLH1*) gene of Males in Cases vs Controls as; Total, Hypermethylation, Non-hypermethylation and Frequency

Table 5: Promoter Region Hypermethylation of Mismatch Repair gene (*hMLH1*) in Females of Case vs Control as; Total, Hypermethylation, Non-hypermethylation and Frequency

PARAMETER	CASE FEMALES (17)	CONTROL FEMALES (08)
HYPERMETHYLATION	08	01
NON-HYPERMETHYLATION	09	07
FREQUENCY	47%	12%

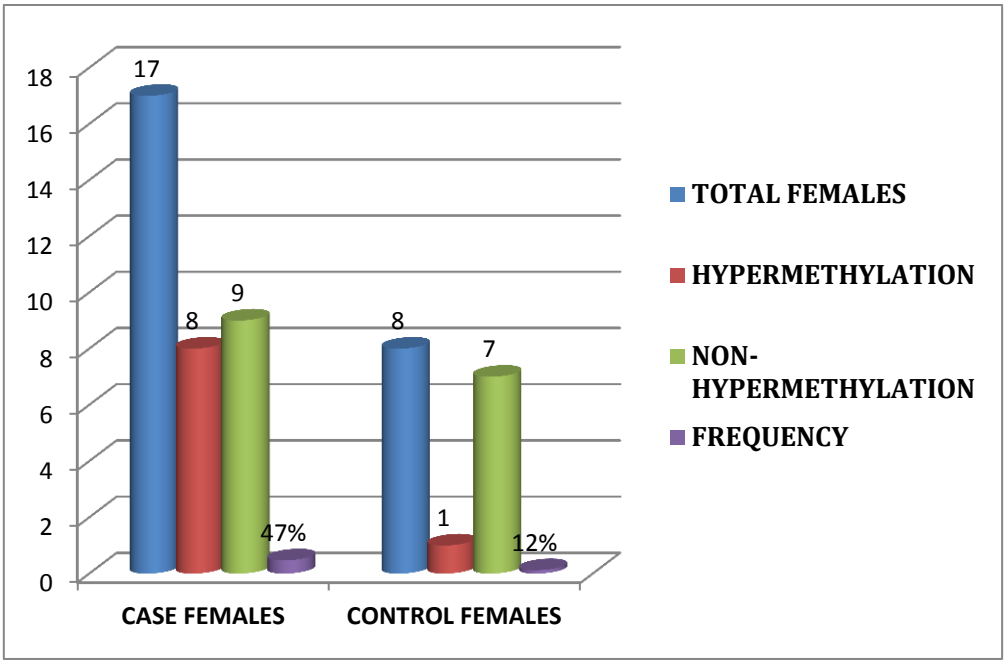

Figure 14: Promoter Region Hypermethylation of Mismatch Repair (*hMLH1*) gene of Females in Cases vs Controls as; Total, Hypermethylation, Non-hypermethylation and Frequency

Table 6: Frequency of promoter region hypermethylation of *hMLH1* gene in esophageal cancer cases vs controls as; Total, Males and Females

PARAMETER	CASE	MALE	FEMALE	CONTROL	MALE	FEMALE
TOTAL	50	33	17	20	12	08
HYPER-METHYLATION	28	20	08	03	02	01
FREQUENCY	56%	60%	47%	15%	16%	12%

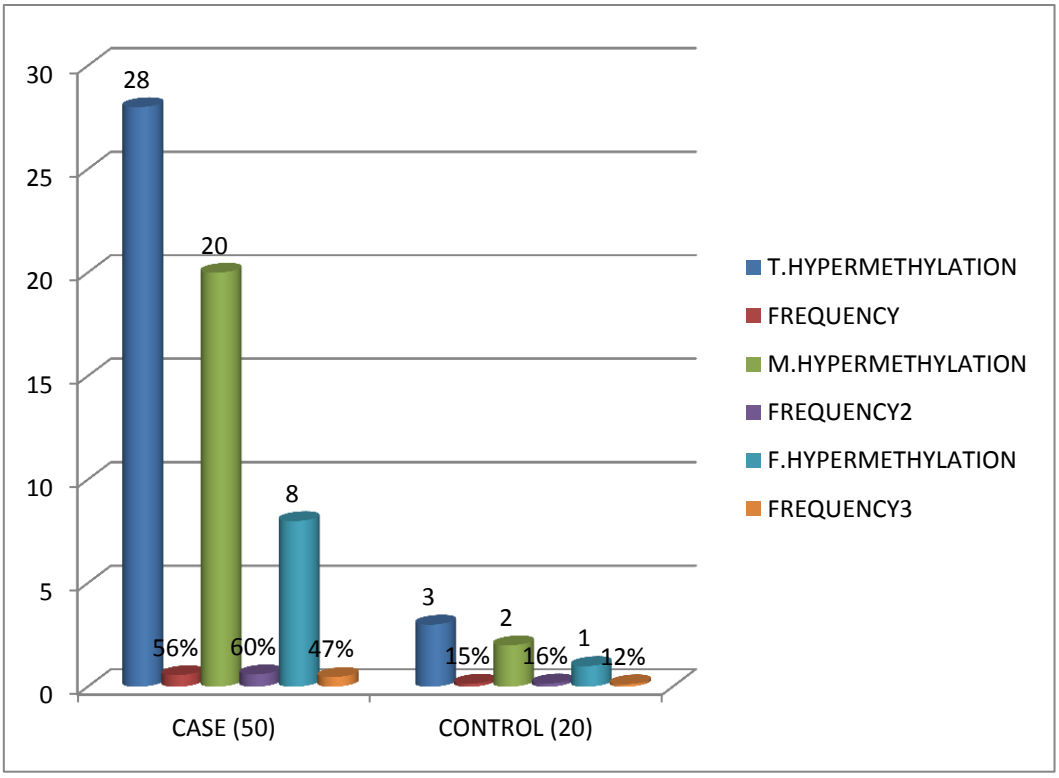

Figure 15: Frequency (% age) Hypermethylation of promoter region of *hMLH1* gene in Esophageal Cancer Cases vs Control as; Total, Males and Females

4.4 FURTHER AS REGARDING THE AGE FIVE MORE SUB-GROUPS OF UPTO-39 YEARS, 40-49 YEARS, 50-59 YEARS, 60-69 YEARS, AND ABOVE 70 YEARS WERE DESIGNED AND REVEALED THE FOLLOWING DATA

4.4a. Upto-39 years of age group

From the data and results it was seen that in this age group, the frequency of hypermethylation of promoter region of Mis-Match Repair Gene MutL homolog 1 (*hMLH1*) in cases was 50% (02 out of 04) while in controls 0% (0 out of 2), and also it was found that Males of this age group shows same frequency 50% (1 out of 2) Compared to Females 50% (1 out of 2), which is shown in histogram in Figure 16 and Data in Table 7.

4.4b. 40-49 years of age group

From the results it was seen that 04 cases of this age group shows promoter region hypermethylation of *hMLH1* Gene out of 06 (66%) while in controls 0 out of 03 (0%) and also it was found that Males of this age group shows high frequency 100% (3 out of 3) Compared to Females 33% (1 out of 3), which is represented in histogram in Figure 17 and Data is Tabulated in Table 8.

4.4c. 50-59 years of age group

From the results and data it was seen that 07 cases of this age group shows promoter region methylation of *hMLH1* gene out of 13 (57%) while in controls 01 out of 05 (20%) and the Males of this age group also shows high frequency 63% (5 out of 8) compared to Females 40% (2 out of 5) of same age group, which is shown in histogram in Figure 18 and data is tabulated in Table 9.

4.4d. 60-69 years of age group

From the results it was seen that 10 cases out of 20 (50%) of this age group showed promoter region hypermethylation of *hMLH1* gene while in controls 02 out of 07 (28%) and the Males of this age group shows lessor frequency 46% (7 out of 15) compared to Females 60% (3 out of 5) which is represented by histogram in Figure 19 and data is tabulated in Table 10.

4.4e. 70-above years of age group

From the results it was seen that 05 cases of this age group shows promoter region methylation of *hMLH1* gene out of 07 cases (71%) while in controls 0 out of 03 (0%) and the Males of the above 70 years of age group shows higher frequency 66% (4 out of 5) compared to Females 50% (1 out of 2) of same age group, which is represented by histogram in Figure 20 and data is tabulated in Table 11.

4.4f. below 40 and above 40 years of age group

The Frequency of MutL homolog 1 (*hMLH1*) gene promoter region hypermethylation was found high in Esophageal Cancer Cases of above 40 years of age, 26 out of 46 (56%) and in controls 03 out of 18 (16%) and was significant as p=0.005 (O.R=6.50 , 95% C.I=1.651-25.58) compared to below 40 years of age 02 out of 04 (50%) and in controls 0 out of 02 (0%) and association was insignificant as p=0.4667 (O.R=5.00 , 95% C.I=0.149-166).

In Case of Males of above 40 years of age hypermethylation frequency was found high 20 out of 31(64%) compared to Males of below 40 years of age 01 out of 02 (50%) and in case of Females of above 40 years of age 07 out of 15(46%) compared to below 40 years of age 01 out of 02(50%).Which is represented by histogram in Figure 21 and data shown in Table 12.

Table 7: Frequency of promoter region hypermethylation of *hMLH1* gene in esophageal cancer cases vs controls of below 40 years of age as; Total, Males and Females

PARAMETER	CASE	MALE	FEMALE	CONTROL	MALE	FEMALE
TOTAL	04	02	02	02	01	01
HYPER-METHYLATION	02	01	01	0	0	0
FREQUENCY	50%	50%	50%	0%	0%	0%

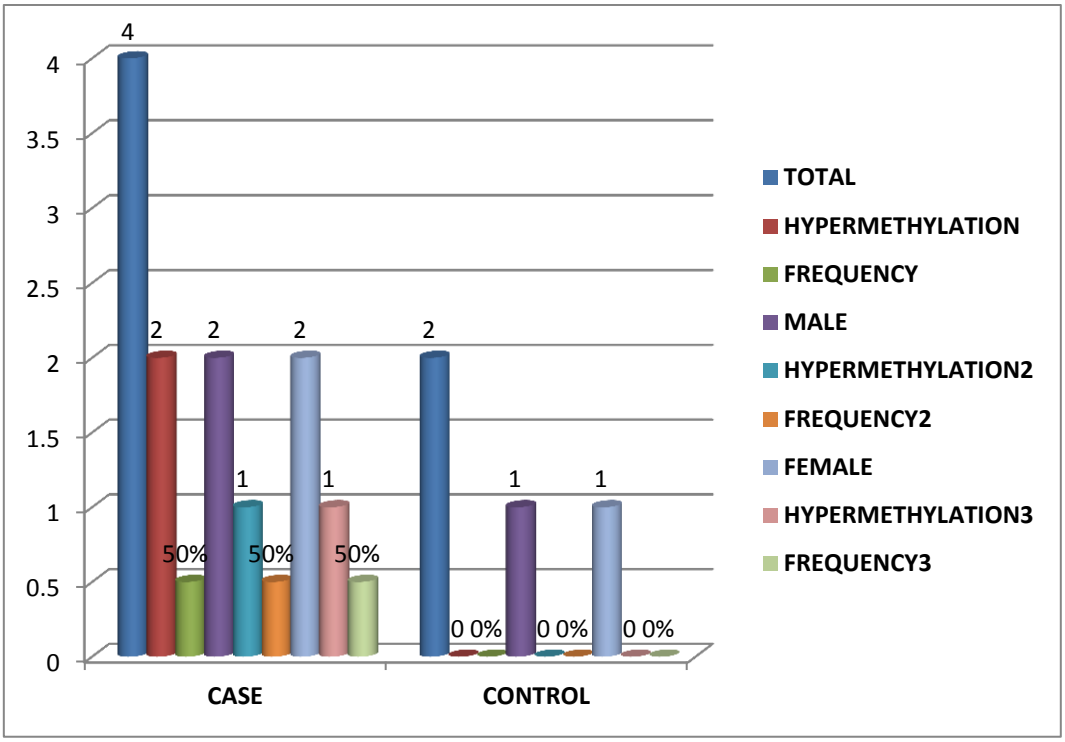

Figure 16: Frequency (%age) Hypermethylation of promoter region of *hMLH1* gene in Esophageal Cancer Cases vs Control of below 40 years of age as; Total, Males and Females

Table 8: Frequency of promoter region hypermethylation of *hMLH1* gene in esophageal cancer cases vs controls of 40-49 years of age group as; Total, Males and Females

PARAMETER	CASE	MALE	FEMALE	CONTROL	MALE	FEMALE
TOTAL	06	03	03	03	02	01
HYPER-METHYLATION	04	03	01	0	0	0
FREQUENCY	66%	100%	33%	0%	0%	0%

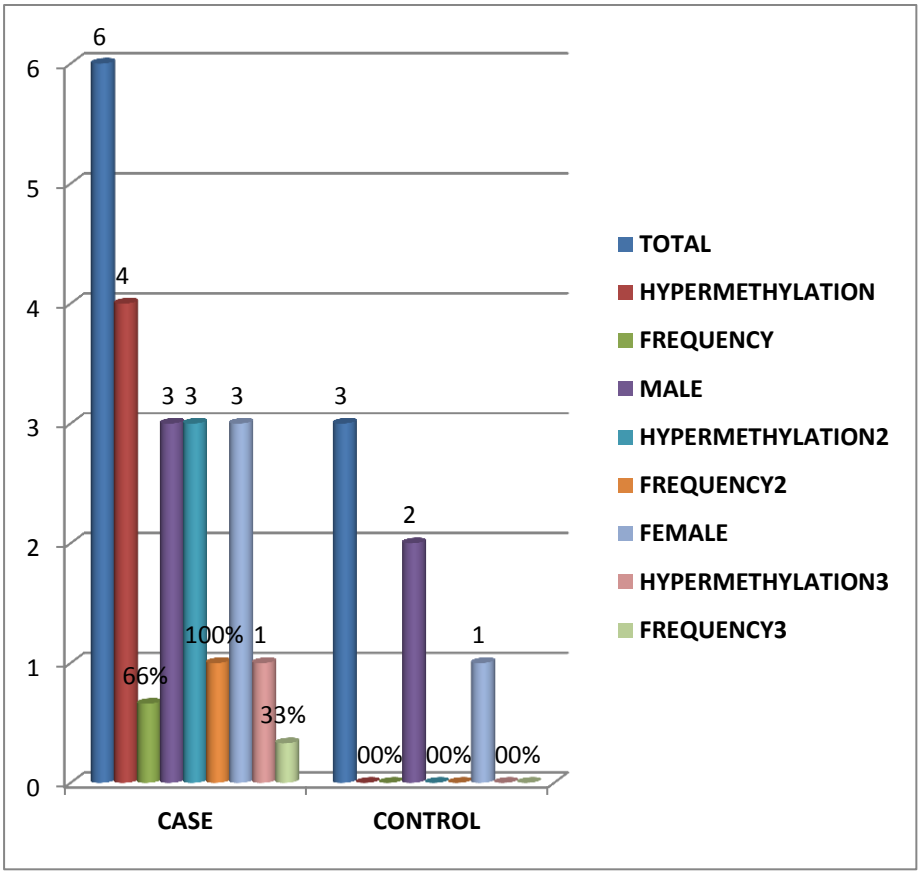

Figure 17: Frequency (%age) Hypermethylation of promoter region of *hMLH1* gene in Esophageal Cancer Cases vs Control of 40-49 years of age group as; Total , Males and Females

Table 9: Frequency of promoter region hypermethylation of *hMLH1* gene in esophageal cancer cases vs controls of 50-59 years of age group as; Total, Males and Females

PARAMETER	CASE	MALE	FEMALE	CONTROL	MALE	FEMALE
TOTAL	13	08	05	05	03	02
HYPER-METHYLATION	07	05	02	01	01	0
FREQUENCY	57%	63%	40%	20%	33%	0%

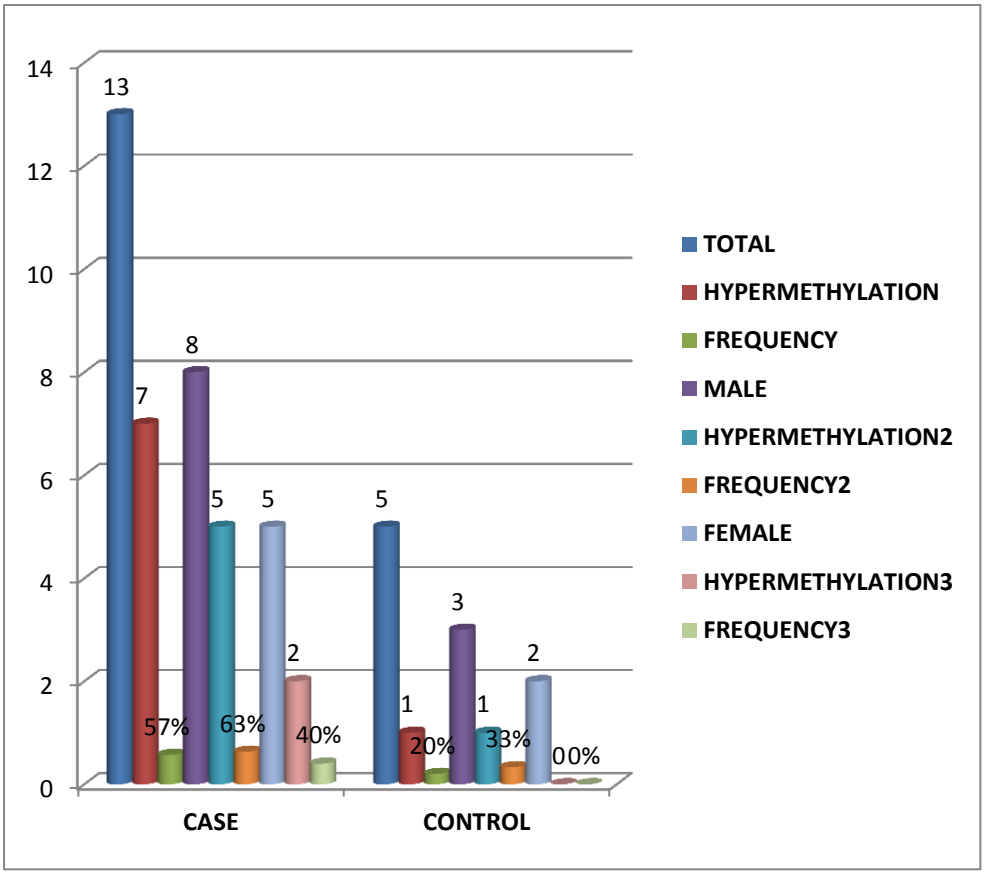

Figure 18: Frequency (%age) Hypermethylation of promoter region of *hMLH1* gene in Esophageal Cancer Cases vs Control of 50-59 years of age group as; Total , Males and Females

Table 10: Frequency of promoter region hypermethylation of *hMLH1* gene in esophageal cancer cases vs controls of 60-69 years of age group as; Total, Males and Females

PARAMETER	CASE	MALE	FEMALE	CONTROL	MALE	FEMALE
TOTAL	20	15	05	07	04	03
HYPER-METHYLATION	10	07	03	02	01	01
FREQUENCY	50%	46%	60%	28%	25%	33%

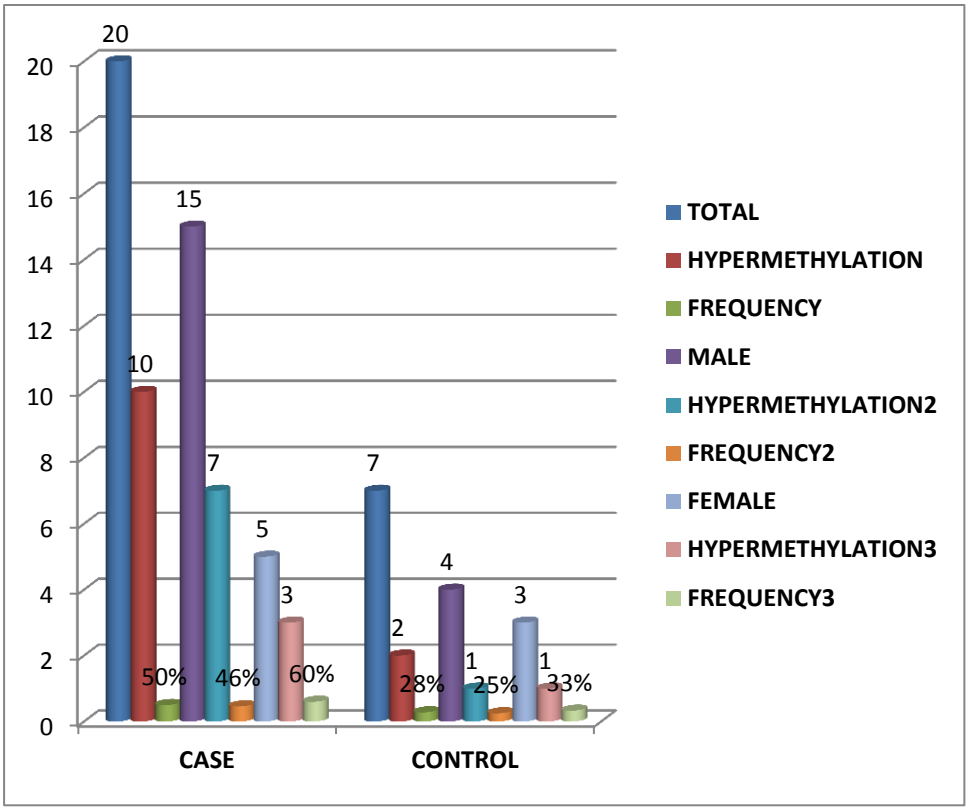

Figure 19: Frequency (%age) Hypermethylation of promoter region of *hMLH1* gene in Esophageal Cancer Cases vs Control of 60-69 years of age group as; Total , Males and Females

Table 11: Frequency of promoter region hypermethylation of *hMLH1* gene in esophageal cancer cases vs controls of 70-years above of age group as; Total, Males and Females

PARAMETER	CASE	MALE	FEMALE	CONTROL	MALE	FEMALE
TOTAL	07	05	02	03	02	01
HYPER-METHYLATION	05	04	01	0	0	0
FREQUENCY	71%	66%	50%	0%	0%	0%

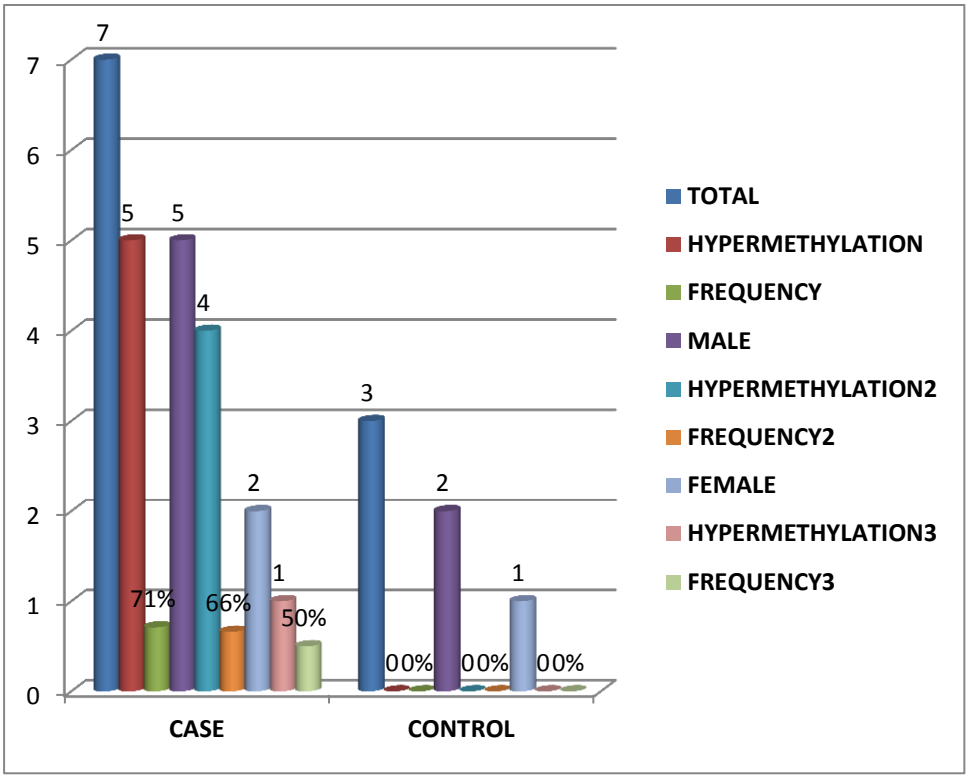

Figure 20: Frequency (%age) Hypermethylation of promoter region of *hMLH1* gene in Esophageal Cancer Cases vs Control of 70 years above of age group as; Total , Males and Females

Table 12: Frequency (%age) of Hypermethylation of promoter region of *hMLH1* gene in Esophageal cancer Cases of Males and Females of below 40 years and above 40 years of age as; Total hypermethylation, Male hypermethylation and Female hypermethylation

PARAMETER		BELOE 40 YRS	ABOVE 40 YRS
CASES	Total	04	46
	Hypermethylation	02	26
	Frequency	50%	56%
CONTROLS	Total	02	18
	Hypermethylation	00	03
	Frequency	0%	16%
MALES	Total	02	31
	Hypermethylation	01	20
	Frequency	50%	64%
FEMALES	Total	02	15
	Hypermethylation	01	07
	Frequency	50%	46%

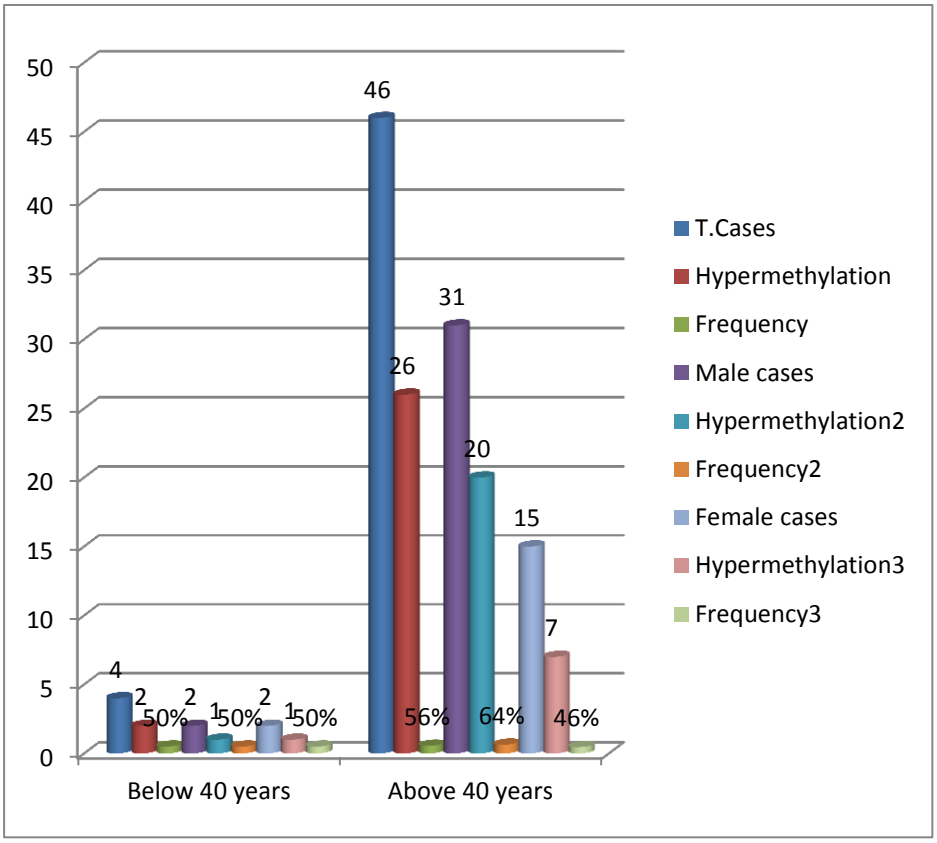

Figure 21: Frequency (%age) of Hypermethylation of promoter region of *hMLH1* gene in Esophageal cancer Cases of Males and Females of below 40 years and above 40 years of age as; Total hypermethylation, Male hypermethylation and Female hypermethylation

DISCUSSION:

Reports indicate that the Kashmir valley of Jammu and Kashmir State of India ranks among the highest incidence area for esophageal cancer in the world (Khuroo *et al.,* 1992). Esophageal cancers are typically carcinomas which arise from the epithelium, or surface lining, of the esophagus. Most esophageal cancers fall into one of two classes: squamous cell carcinomas, which are similar to head and neck cancer in their appearance and association with tobacco and alcohol consumption, and adenocarcinomas, which are often associated with a history of gastro esophageal reflux disease and Barrett's esophagus. A general rule of thumb is that a cancer in the upper two-thirds is a squamous cell carcinoma and one in the lower one-third is an adenocarcinoma (Shield *et al.,* 2005; Halperin *et al.,* 2008).Injury or irritation to the esophagus is caused by a number of substances and may result in esophagitis, GERD, Barrett's esophagus or esophageal cancer. At the outset, most of these conditions exhibit an inflammatory pathology, which may persist as a benign lesion or progress to cancer. Nearly 60% of severe grade dysplasias and 5-20% of cases of achalasia in the esophagus progress to malignancy (Peracchia *et al.,* 1991; Meijssen *et al.,* 1992). The risk of Barrett's esophagus developing into invasive carcinoma is 40- fold higher than the cancer risk of the general population (Cameron *et al.,* 1985). Molecular markers are therefore needed to indicate the risk of progression of these inflammatory conditions to more severe diseases including malignancy. These markers should also be able to predict the prognosis of cancer, so that we can assess survival chances and response to treatment, and plan a suitable management regime. 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.

Genetic mutation of genes that inhibit the formation of tumors has long been known to be one of the main driving forces in the development of cancer. However, recent data have focused our attention to the contribution of epigenetics to tumorigenesis. In tumorigenesis of the esophagus, the epigenetic inactivation of genes is as an important a driving force as the inactivation of genes by mutation (Enzinger and Mayer 1993; Chen and Yang 2001; Momparler 2003). Synergistic effect of dietary, environmental, genetic and microbial factors is being associated with the development of esophageal cancer and it has multifactorial epidemiology (Zhou and Watanabe 1999; Zhang *et al*., 2000; Nayar *et al*., 2000; Shi *et al*., 2000; Lagergren *et al*., 2000; Dhillon *et al*., 2001). 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 (Parkin *et al*., 1988; Shottenfeld, 1984). Kashmir is a known region of high incidence of esophageal cancer (Maqbool and Ahad 1974; Jan and Zargar 1988; Khuroo 1992) 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 (Parkin *et al*., 2005).

DNA methylation in cancer has become the topic of intense investigation. As compared with normal cells, the malignant cells show major disruptions in their DNA methylation patterns (Baylin and Herman 2000). Hypermethylation involves CpG islands whereas Hypomethylation usually involves repeated DNA sequences, such as long interspersed nuclear elements (Ehrlich, 2002). Promoter hypermethylation is an alternative mechanism of gene inactivation in carcinogenesis (Baylin *et al.*, 1998). Several studies have suggested that aberrant methylation of the promoter causes transcriptional silencing of some important suppressor genes, such as p16 (Merlo *et al*., 1995), E-cadherin (Graff *et al*., 1995), and von Hippel Lindau (VHL) gene (Herman *et al*., 1994), and this has been implicated in the carcinogenic process in many cancers (Baylin *et al.*, 1998).

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 (Modrich, 1991). Dysfunction of a mismatch repair system such as *hMLH1* and *hMSH2* could alter microsatellites, short tandem repetitive sequences (Thibodeau *et al*., 1993). The mismatch repair system is composed of a highly diverse group of proteins that interact with numerous DNA structures during DNA repair and replication (Hoffmann and

Borts 2004). Mismatch repair (MMR) plays a central role in maintaining genomic stability by repairing DNA replication errors and inhibiting recombination between non-identical sequences (Jacob and Praz 2002; Duval and Hamelin 2002). Loss of mismatch repair causes destabilization of the genome and results in high mutation frequency (Loukola *et al*., 2001). *hMLH1* gene is a prominent component in the human mismatch repair system and its dysfunction is involved in a number of patients with HNPCC (Han *et al*., 1995; Hutter *et al*., 1995). It has been reported that germline mutations of MMR are identified in nearly 80% of the patients with HNPCC and almost 60% of the mutations are in *hMLH1* (Peltomaki and Vasen 1997). 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 (Clark and Melki 2002). 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 (Jones and Baylin 2002).

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 the present study, we investigated the level of *hMLH1* promoter methylation in esophageal carcinoma tissues of patients from Kashmir valley where frequency is higher as compared to other regions of India. Cancer and some chronic inflammatory conditions have been associated with genomic instability (Ishitsuka *et al.,* 2001; Gao *et al.,* 2005). The MMR system maintains genomic integrity, and it is accepted that defects in MMR genes are responsible for the microsatellite instability (MSI) observed in different diseases (Brentnall *et al.,* 1995; Lynch and Hoops 2002). Approximately 27% of esophageal tumors deficient in *hMLH1* repair gene expression have been associated with MSI (Hayashi *et al.,* 2003). It was also observed *hMLH1* hypermethylation to be associated with MSI in about 42% of esophageal cancers (Vasavi and Hasan Q).MMR gene silencing has been reported in several cancers and inflammatory conditions such as inflammatory bowel disease, head and neck cancers, and cancers of the GI tract (Wang *et al.* 2001; Bubb *et al.,* 1996; Kang *et al.,* 2002). In most of these studies, immunohistochemistry and mRNA

expression analysis showed the absence of *hMLH1* protein; however, no mutations were found to be responsible for this (Kane *et al.,* 1997; Liu *et al.,* 1995). Epigenetic silencing through promoter methylation of the *hMLH1* repair gene was associated with loss of *hMLH1* protein expression (Razin *et al.,* 1980; Kane *et al.,* 1997). In esophageal cancers, there are controversial reports regarding the association of hypermethylation with *hMLH1* gene silencing (Hayashi *et al.,* 2003; Nie *et al.,* 2002). Esophageal cancers have been associated with late diagnosis and poor prognosis.

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. The Study included 33 Male Cases and 17 Female Cases out of which only 04 Cases were below the age of 40 years and remaining 46 Cases were above the age of 40 years and Controls include 12 Males and 08 Females out of which 02 Controls were below the age of 40 years and remaining 18 Controls were above the age of 40 years.

Figure 10 and 11 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* (Hayashi *et al.,* 2003; Geddert *et al.,* 2004; Nie *et al.,* 2002). 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 conducted by Geddert *et al., 2004*; Hayashi *et al., 2003;* Nie *et al., 2002*, paraffin-embedded tissue material was used, while in the present study we employed fresh biopsy specimens. Wang *et al., 2003* reported a higher percentage of *hMLH1* hypermethylation with *Hpa*II-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 10). 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 10). 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 11 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 11 control 67). Statistically the association of promoter region hypermethylation of mismatch repair gene *(hMLH1*) with esophageal cancer was evaluated using χ^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). It was also found that the methylation status of MutL homolog 1 *(hMLH1*) gene in esophageal cancer cases was high in Males (60%) compared to Females (47%) and in Controls, Males (16%) also shows high with respect to Females (12%) which shows insignificant association as p=0.3827,odds ratio=1.7310 and 95% C.I=0.5312-5.639.From the data it was concluded that the Frequency of MutL homolog 1 (*hMLH1*) gene promoter region hypermethylation was found high in Esophageal Cancer Cases of above 40 years of age, 26 out of 46 (56%) and in controls 03 out of 18 (16%) and was significant as p=0.005 (O.R=6.50 , 95% C.I=1.651-25.58) compared to below 40 years of age 02 out of 04 (50%) and in controls 0 out of 02 (0%) and association was insignificant as p=0.4667 (O.R=5.00 , 95% C.I=0.149-166) and was evaluated by Fishers exact test.

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.

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. This became more apparent when the data for hypermethylation was interpreted taking gender into consideration here it was seen that males shows higher frequency of promoter region hypermethylation as compared to females which was earlier reported in literature and also patients of above 40 years of age shows high frequency compared to below 40 years of age.

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. In addition, the fact that methylation can be reversed *in vitro* and the effect of the demethylating agent 5-aza-2′-deoxycytidine *in vitro* raise hope for new treatment strategies for esophageal cancer patients. Furthermore, understanding of the significance of aberrant DNA methylation in the precancerous stage may show that a new strategy, correction of aberrant DNA methylation, can prevent esophageal cancer in people with premalignant lesions, such as Barrett's esophagus, basal cell hyperplasia (BCH) and dysplasia (DYS).

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.

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APPENDIX:

CHEMICALS AND REAGENTS

Chemicals:

Chemical Name	**Company**
Absolute ethanol	BENGAL CHEMICALS
Acetone	GALAXO LABORATORIES
Agarose	MP BIOMEDICALS
Ammonium chloride	BDH
Ammonium acetate	BDH
Bromophenol blue	SARABHAI M CHEMICALS
Chloroform	THOMAS BAKERS
De Ionized water	ALFA LABORATORIES
Ethedium bromide	SRL
Ethyl acetate	MERCK
Ethylene diamine tetra acetate	LOBA CHEMIE
Formaldehyde	GALAXO LABORATORIES
Glacial acetic acid	MERCK
Hydrochloric acid	S D FINE CHEMICALS
Hydrogen peroxide	MERCK
8-Hydroxyquinoline	CDH
Isoamyl alcohol	BDH
Isopropanol	THOMAS BAKERS
Magnesium chloride	MERCK
Methanol	SARABHAI M CHEMICALS
Phenol	SRL
Potassium acetate	QUALGENS
Potassium bicarbonate	QUALGENS
Potassium chloride	LOBA- CHEMIE
Potassium hydroxide	S D FINE CHEMICALS
2-Propanol	MERCK
Sodium acetate	SARABHAI M CHEMICALS
Sodium azide	LOBA CHEMIE
Sodiun bisulphate	LOBA CHEMIE

Sodium carbonate	FIZMERCK
Sodium chloride	MERCK
Sodium dodecyl sulphate	MP BIOMEDICALS
Sodium hydroxide	HEMEDIA
Sodium hydrogen carbonate	LOBA- CHEMIE
Sodium phosphate dibasic	LOBA- CHEMIE
Sodiun thiosulfate	LOBA CHEMIE
Sucrose	QUALGENS
Sulfuric acid	MERCK
TE buffer	SRL
Tris base	SIGMA CHEMICAL COMPANY
Tris HCL	HIMEDIA
Triton X 100	S D FINE CHEMICALS

Enzymes:

Taq polymerase	FERMENTAS / BIOTOOLS
Proteinase K	ZYMO RESEARCH

MISCELLANEOUS MATERIAL:

100bp DNA ladder	FERMENTAS / BIO ENZYME

PCR Reagents:

10 X Buffer (with $Mgcl_2$)	BIOTOOLS
dNTPs	CINNAGEN
Primers (methylated and unmethylated)	GENESCRIPT
Universal Methylated Human DNA Standard and Control with primers	ZYMO RESEARCH

DNA Isolation:

DNA was iosolated by kit based method. The kit used was Quick- g DNA^{TM} MiniPrep supplied by ZYMO RESEARCH. The protocol followed was as directed by the company.

DNA storage buffer:

0.5 M EDTA	0.01 ml
1 M Tris	0.5 ml

Final volume was made 50 ml with sterile distilled water.

DNA Bisulfite Modification:

DNA was modified by kit based method, the kit used was EZ DNA Methylation™ Kit supplied by ZYMO RESEARCH. The protocol followed was as directed by the company.

Reagents for Agarose Gel Electrophoresis:

Agarose 1%:

Agarose	0.5g
Buffer	50ml
EtBr	10µl

Agarose was dissolved in a buffer and heated till a clear solution is formed. EtBr was then added to the solution during its cooling just before being poured into the casting tray.

Bromophenol Blue:

Bromophenol Blue	0.4g
Sucrose	20.og

Bromophenol blue was dissolved in 100ml of distilled water.

From the above stock solution 31.25ml was taken and sucrose was added. Final volume was made 50ml with distilled water.

Ethedium Bromide:

Ethedium bromide	10mg

Ehedium Bromide was dissolved in 1ml of distilled water. The solution was stored in a dark bottle at 4˚C.

50X TAE (pH 8.0) Stock Solution:

Tris base	242g
0.5M	100ml
Glacial acetic acid	57.1ml

Final volume was made 1000ml with distilled water. This is stock solution.

1X TAE (pH 8.0) Working Solution:

50 X TAE	20ml

Final volume was made 1000ml with distilled water.

Reagents for PCR:

Stock:

Deoxy ribose nucleotide triphosphate (dNTP) 100mM each dATP, dGTP, dCTP and dTTP.

Taq polymerase (5U/ml)

10X Taq buffer (16 mmol/L ammonium sulfate, 67 mmol/L Tris- HCL, pH 8.8, 10 mmol/L 2-mercaptoethanol), 6.7 mmol/ L MgCl2)

Primers: 100pM in sterile deionised water (Genescript)

100bp DNA ladder (0.5µg/µl)