Elucidation of etiology of colorectal cancer: A study on silencing of *p16* gene by promoter hypermethylation

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

By

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UNDER THE SUPERVISION OF

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CERTIFICATE

Certified that the work embodied in the dissertation entitled **"Elucidation of etiology of colorectal cancer: A study on silencing of *p16* gene by promoter hypermethylation"** is the bonafide work of Mr. Hilal Ahmad Wani and has been carried out under the joint supervision of Dr. Sabhiya Majid (H.O.D Department of Biochemistry, Govt. Medical College Srinagar), Dr. Shajrul Amin (Department of Biochemistry, University of Kashmir) and Dr. Nisar A. Wani (Department of Surgery, Govt. Medical College Srinagar) in the Department of Biochemistry, Govt. Medical College Srinagar and Department of Biochemistry, University of Kashmir. The work is suitable for the award of M.Phil degree in Biochemistry.

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DECLARATION

I, Hilal Ahmad Wani, declare that the work embodied in this dissertation entitled **"Elucidation of etiology of colorectal cancer: A study on silencing of *p16* gene by promoter hypermethylation"** has been carried out by me in the Department of Biochemistry, Government Medical College, Srinagar (Research center University of Kashmir) and Department of Biochemistry, University of Kashmir, Srinagar and is original. The work embodies the results of my observations which are advancement to the previous knowledge in the subject.

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Dated:

Abbreviations:

µg	Microgram
µl	Microlitre
µM	Micromolar
ACS	American cancer society
AJCC	American Joint Committee on Cancer
AN	Almost negligible
ARF	Alternate open reading frame
bp	Base pair
BPB	Bromophenol blue
C.I	Class Interval
CDK	Cyclin dependent kinase
CEA	Carcinoembryonic antigen
CRC	Colorectal Cancer
DDW	Double distilled water
DMH	Dimethylhydrazine
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide –triphosphate
DRE	Digital rectal exam
EDTA	Ethylene diaminetetraacetic acid
EIF	Eukaryotic initiation factor
ESCC	Esophageal squamous cell carcinoma
EtBr	Ethidium bromide
Fig.	Figure
FOBT	Fecal occult blood test
GC	Gastric Cancer

GIT	Gastrointestinal tract
gm	Grams
HNPCC	Hereditary non polyposis colorectal cancer
Kb	Kilobase pair
Kd	Kilo Dalton
LCA	Lithocholic acid
M	Molar
MBD	Methyl Binding Domain
mg	Miligram
min	Minutes
ml	Mililitre
mM	mili molar
mRNA	messenger ribonucleic acid
MSP	Methyl Specific polymerase Chain Reaction
NaCl	Sodium chloride
NCBI	National Centre for Biotechnology Information
NCI	National Cancer Institute
ng	Nanogram
NOCs	Nitrosoamine carcinogens
O.D	Optical density
O.R	Odds ratio
^{o}C	Degree Celsius

OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
pmol	pico mole
Rb	Retinoblastoma
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
sec	Second
SKIMS	Sheri- Kashmir Institute of Medical Sciences Soura
SNP	Single nucleotide polymorphism
ST	Salted tea
STE	Sodiumchloride-Tris-Ethylenediaminetetraacetic acid
TAE	Trisacetic EDTA
Taq	*Thermus aquaticus* DNA polymerase
TBE	Tris-borate-EDTA
Tm	Melting temperature
TNM	Tumour node metestasis
Tris	Tris (hydroxymethylaminomethane)
UV	Ultraviolet
WHO	World Health Organisation
XME	Xenobiotic metabolizing enzyme

Abstract:

Colorectal cancer (CRC), commonly known as bowel cancer is the third most common cause of cancer-related deaths in the western world. Colorectal cancer is one of the leading malignancies worldwide. CRC has been reported to show geographical variation in its incidence, even within areas of ethnic homogeneity. The usual treatment is surgery and subsequent chemotherapy and radiotherapy. Cancer development and progression is dictated by series of alterations in genes such as tumor suppressor genes, DNA repair genes, oncogenes and others. In colorectal carcinogenesis disturbances different from mutations called an epigenetic regulation are also taken into consideration.

The aim of this study was to study the promoter hypermethylation of CpG islands of *p16* gene in colorectal cancer patients among the Kashmiri population. The study included 70 surgically obtained colorectal samples among which 50 were obtained from colorectal cancer patients and 20 were histopathologically normal colorectal samples. All the samples were histopathologically confirmed before further processing. DNA was extracted from all the samples and was modified using bisulphite modification kit. Methylation-specific polymerase (MSP) chain reaction was used for analysis of the promoter hypermethylation status of *p16* gene. The genetic analysis of the cases and controls by MSP- PCR method, for checking the promoter hypermethylation of CpG islands of *p16* gene revealed that unlike other high risk regions, Kashmiri population has a different promoter hypermethylation profile of *p16* gene. 66% of the cases showed *p16* promoter hypermethylation while as 34% of the cases were nonhypermethylated. The study also revealed that 20% of the normal cases also had promoter hypermethylation of *p16* gene and 80% did not showed promoter hypermethylation of *p16* gene. The association of promoter hypermethylation with colorectal cancer was evaluated by $\chi 2$ (Chi square) test with Odds ratio and was found to be significant. Among 29 male cases and 21 female cases, the association of promoter hypermethylation with colorectal cancer was evaluated using Fisher's exact test and was found to be significant in both males and females. Occurrence of *p16* promoter hypermethylation was found to be unequally distributed in males and females with more frequency in males than in females but the difference

was not statistically significant. When the frequency of *p16* promoter hypermethylation was compared with clinical staging of the disease, *p16* promoter hypermethylation was found to be certainly higher in Stage III/IV (83.33%) compared to Stage I/ II (56.25%) but the difference was not statistically significant. Also, the degree of *p16* promoter hypermethylation increased with the increasing severity of the lesion but the difference was not again statistically significant.

These results clearly suggest that *p16* aberrant promoter hypermethylation in Kashmiri population contributes to the process of carcinogenesis in colorectal cancer and is reportedly one of the commonest epigenetic changes in the development of human CRC. It also demonstrates that hypermethylation of *p16* gene can be designated as epigenetic biomarker for the screening, diagnosis and prognosis of colorectal cancer. The data gives a clue that *p16* gene expression can be readily and fully restored and growth rate of cancer cells decreased by treatment of cancer cells with demethylating agents and DNA methylation inhibitors.

Introduction:

1.1. COLORECTAL CANCER:

Colorectal cancer, also called colon cancer or large bowel cancer includes cancerous growths in the colon, rectum and appendix. With 6,55,000 deaths worldwide per year, it is the fifth most common form of cancer in the United States and the third leading cause of cancer-related deaths in the Western world (World Health Organization, 2006 and National Cancer Institute, 2009). Colorectal cancer being the commonest cancer is the major cause of mortality and morbidity worldwide, there are nearly one million new cases of colorectal cancer diagnosed world-wide each year and half a million deaths (Boyle and Elena, 2002). It is a commonly diagnosed cancer in both men and women. In 2008, about 1, 48, 810 new cases were diagnosed in U.S and almost 49,960 deaths from colorectal cancer were speculated (Cancer Facts and Figures, 2008). The incidence of this malignancy shows considerable variation among racially or ethnically defined populations in multiracial/ethnic countries. Kashmir has been reported by now as a high-incidence area of GIT cancers (Shah *et al.,* 1990; Mir *et al.,* 2005; Salam *et al.,* 2009). Colorectal Cancer in Kashmir valley is the third most common GIT cancer after esophageal and gastric cancer (Sameer *et al.,* 2009). The development of CRC is a multistep process, which can arise due to the cumulative effect of mutations in various different proto-oncogenes, tumor suppressor genes, and/or from epigenetic changes in DNA (Vogelstein *et al.,* 1988; Fearon *et al.,* 1990; Mustafa *et al.,* 2007). Colorectal cancer is a disease originating from the epithelial cells lining the colon or rectum of the gastrointestinal tract, as a result of mutations to some of the genes. Some of the mutations are inherited, and others are acquired (Ionov *et al.,* 1993; Chakravarthi *et al.,* 1999). Some genes are oncogenes - they are over expressed in colorectal cancer. *RAS*, *RAF*, and *PI3K*, which normally encourage the cell to divide in response to growth factors, can become mutated with mutations that make them over signal the cell. There are two major mechanisms of gene inactivation. One is the genetic mechanism, i.e. the aberration of DNA structure such as homozygous deletion or intragenic mutation resulting in the gene inactivation. The other is the epigenetic mechanism, i.e., the methylation of the position 5 of cytosine (C) leading to the

lack of gene expression, while the structure and the product of the gene remain unchanged.

DNA methylation is a naturally occurring event in both prokaryotic and eukaryotic organisms. In prokaryotes DNA methylation provides a way to protect host DNA from digestion by restriction endonucleases that are designed to eliminate foreign DNA, and in higher eukaryotes DNA methylation functions in the regulation/control of gene expression (Costello *et al.,* 2001). It has been demonstrated that aberrant DNA methylation is a widespread phenomenon in cancer and may be among the earliest changes to occur during oncogenesis (Stirzaker, 1997). DNA methylation has also been shown to play a central role in gene imprinting, embryonic development, chromosome gene silencing, and cell cycle regulation. In many plants and animals, DNA methylation consists of the addition of a methyl group to the fifth carbon position of the cytosine pyrimidine ring via a methyltransferase enzyme (Adams, 1995). The majority of DNA methylation in mammals occurs in 5'-CpG-3' dinucleotides, but other methylation patterns do exist. In fact, about 80 percent of all 5'-CpG-3' dinucleotides in mammalian genomes are found to be methylated, whereas the majority of the 20 percent that remain unmethylated are within promoters or in the first exons of genes.

In higher order eukaryotes, DNA is methylated only at cytosines located 5' to guanosine in the CpG dinucleotide and this modification has important regulatory effects on gene expression, especially when involving CpG rich areas known as CpG islands, located in the promoter region of many genes. While almost all gene-associated islands are protected from methylation on autosomal chromosomes, extensive methylation of CpG islands has been associated with transcriptional inactivation of selected imprinted genes and the genes on inactivated X chromosome of females. Aberrant methylation of normally unmethylated CpG islands has been associated with transcriptional inactivation of gene (Zingg *et al.,* 1997). It is a complex process catalyzed by DNA methyltransferases (Dnmts). The methyl group is donated by the universal methyl donor s-adenosyl L -methionine. Unmethylated CpGs are grouped in clusters called CpG islands that are present in the 5' regulatory regions of many genes. In many disease processes such as cancer, gene promoter CpG islands acquire abnormal hypermethylation, which results in

transcriptional silencing (Jaenisch *et al.*, 2003). DNA methylation may affect the transcription of genes in two ways. First, the methylation of DNA may itself physically impede the binding of transcriptional proteins to the gene and secondly, and likely more important, methylated DNA may be bound by proteins known as methyl-CpG-binding domain proteins (MBDs). MBD proteins then recruit additional proteins to the locus, such as histone deacetylases and other chromatin remodeling proteins that can modify histones, thereby forming compact, inactive chromatin termed silent chromatin. Promoter hypermethylation is one of the hallmarks of carcinogenesis associated with transcriptional silencing and loss of expression of genes encoding for diverse cellular pathways (Esteller, 2007). Most of the evidence exists for tumor suppressor genes (Issa, 2004; Laird, 2005). Hypermethylation leads to silencing of gene transcription through a complex process involving chromatin condensation and histone deacetylation (Jones *et al.,* 1998; Wade *et al.,* 1999).

The ability to detect and quantify DNA methylation efficiently and accurately has become essential for the study of cancer, gene expression, genetic disease, as well as many other important aspects of biology. To date a number of methods have been developed to detect/quantify DNA methylation including: high- performance capillary electrophoresis (Fraga *et al.,* 2000) and methylation-sensitive arbitrarily primed PCR (Gonzalgo, 1997). However, the most common technique used today remains the bisulfite conversion method (Frommer, 1992).This technique involves treating methylated DNA with bisulfite which converts unmethylated cytosines into uracil. Methylated cytosines remain unchanged during the treatment. Once converted the methylation profile of the DNA can be determined by PCR amplification followed by DNA sequencing.

1.2. *P16* GENE

Epigenetic silencing through DNA methylation can begin very early in tumor progression and may affect multiple genes involved in different cellular pathways including cell cycle control, DNA repair and many others (Baylin *et al.,* 1998; 2001). Promoter hypermethylation can cause transcriptional inactivation of tumor suppressor gene like *p16*. Human *p16* gene possesses a CpG island in the promoter region and it has been reported that the

methylation of discrete regions of the *p16* CpG island is associated with the silencing of the gene. Promoter hypermethylation of *p16* gene in colorectal cancer patients has been studied and documented in several studies. Some studies have suggested that *p16* plays an important role in cancer pathogenesis and has implications for improving the clinical management (Lam *et al.,* 2008). Methylation of the *p16* (*INK4a*) gene contributes to the process of carcinogenesis in colorectal cancer and is useful as a prognostic factor in the early stage (Yi Jing *et al.,* 2001; Ishiguro *et al.,* 2006). *P16* might act as a tumor suppressor in colorectal carcinomas and was more frequently methylated in advanced colorectal carcinomas (Hibi *et al.,* 2005; Goto *et al.,* 2008). *P16* hypermethylation plays a role in the carcinogenesis of colorectal cancers (Liang *et al.,* 1999). The *p16* (*CDKN2a*/*INK4a*) gene is an important tumor-suppressor gene and it is located on human chromosome 9 in the region 9p21, involved in the *p16/cyclin dependent kinase/retinoblastoma* gene pathway of cell cycle control, in which the *p16* protein is considered to be a negative regulator involved in the inhibition of G1 phase progression (Rocco *et al.,* 2001). Since its discovery as a CDKI (cyclin-dependent kinase inhibitor) in 1993, the tumor suppressor *p16* has gained widespread importance in cancer. It is also called as *INK4A* or *MTS-1* or *CDKN2A*. Different alternatively spliced gene products are encoded by *p16*: Protein *P16* and p14ARF Protein. The human *p16* protein is a 16-KD protein containing 156 amino acids and was first discovered in a yeast two-hybrid system to detect proteins that interact with human cyclin Dependent kinase (Ruas *et al.,* 1998). Its mechanism of action as a CDKI has been elegantly elucidated and involves binding to and inactivating the cyclin D-cyclin-dependent kinase 4 (or 6) complex, and thus renders the retinoblastoma protein unphosphorylated and inactive. This effect blocks the transcription of important cell-cycle regulatory proteins that promote passage of the cell through the restriction point of the G1 stage and results in cell-cycle arrest (Liggett *et al.,* 1998). Cyclin-dependent kinase inhibitor 2A (*p16* inhibits CDK4), also known as CDKN2A, is a tumor suppressor protein, which in humans is encoded by the *CDKN2A* gene having three exons (Entrez Gene; Nobori *et al.,* 1994; Stone *et al.,* 1995). *P16* plays an important role in regulating the cell cycle, and mutations in *p16* increase the risk of developing a variety of cancers, notably melanoma. *P16* inactivation

breaks down the regulatory mechanism of the cell cycle. As a tumor suppressor gene, being silenced by any mechanism will promote carcinogenesis. This gene generates several transcript variants which differ in their first exons. At least three alternatively-spliced variants encoding distinct proteins have been reported, two of which encode structurally related isoforms known to function as inhibitors of CDK4 kinase. The remaining transcript includes an alternate first exon located 20 Kb upstream of the remainder of the gene; this transcript contains an alternate open reading frame (ARF) that specifies a protein which is structurally unrelated to the products of the other variants. This ARF product functions as a stabilizer of the tumor suppressor protein p53 as it can interact with, and sequester, MDM2, a protein responsible for the degradation of p53 (Molecular biology of cancer, Oxford University Press, 2005). In spite of the structural and functional differences, the CDK inhibitor isoforms and the ARF product encoded by this gene, through the regulatory roles of CDK4 and p53 in cell cycle G1 progression, share a common functionality in cell cycle G1 control. This gene is frequently mutated or deleted in a wide variety of tumors, and is known to be an important tumor suppressor gene. Increased expression of the *p16* gene as organisms age reduces the proliferation of stem cells (Krishnamurthy *et al.,* 2006). This reduction in the division and production of stem cells protects against cancer while increasing the risks associated with senescence. Mutations in the *CDKN2A* gene are associated with increased risk of a wide range of cancers and alterations of the gene are frequently seen in cancer cell lines. Pancreatic adenocarcinoma is often associated with mutations in the *CDKN2A* gene (Caldas *et al.,* 1994; Bartsch *et al.,* 1995; Liu *et al.,* 1995). Homozygous deletion of *p16* is frequently found in esophageal cancer and gastric cancer cell lines (Igaki *et al.,* 1994). Concentrations of p16INK4a increase dramatically as tissue ages. Therefore p16INK4a could potentially be used as a blood test that measures how fast the body's tissues are aging at a molecular level (Liu *et al.,* 2009). The inactivation of *p16INK4a* gene due to aberrant promoter hypermethylation in esophageal, lung, gastric and hepatocellular carcinoma has been well documented (Hibi *et al.,* 2001; Huang *et al.,* 2004; Qing *et al.,* 2005; Liu *et al.,* 2006; Belinsky *et al.,* 2006; Abbaszadegan *et al.,* 2008; Salam *et al.,* 2009). However, before accepting the conclusion that hypermethylation

of tumor suppressor gene promoter is invariably the cause of gene inactivation, it is worth evaluating the data a bit more critically. Epigenetic mechanisms of gene inactivation, including promoter hypermethylation, are undoubtedly important in cancer development and represent an alternative means of inactivating tumor suppressor genes. Nevertheless, the standard of proof for establishing that hypermethylation of the promoter of any given gene has a critical role in loss of gene expression and cancer development should probably be set quite high, regardless of whether the gene is a well-established tumor suppressor gene, like *p16*, or a potential tumor suppressor gene.

There are contradictory reports regarding *p16* promoter hypermethylation in the etiology of various cancers including colorectal cancer. The present study is an attempt to analyze the *p16* gene promoter hypermethylation in colorectal carcinoma in Kashmiri population. As this aspect has not been well studied in colorectal carcinoma patients of Kashmir valley. So the study was confined to study the promoter hypermethylation of *p16* gene in colorectal patients of Kashmir, which may help in prognosis and diagnosis of the disease so that further preventive measures could be taken.

Review of literature:

2.1 Cancer

Cancer, medically called a malignant neoplasm, is a term for a large group of different diseases. In cancer, cells divide and grow uncontrollably, forming malignant tumors, and invade nearby parts of the body. The cancer may also spread to more distant parts of the body through the lymphatic system or bloodstream. Not all tumors are cancerous. Benign tumors do not grow uncontrollably, do not invade neighbouring tissues, and do not spread through the body. Healthy cells control their own growth and will destroy themselves if they become unhealthy. Cell division is a complex process that is normally tightly regulated. Cancer happens when problems in the genes in a cell prevent these controls from working. These problems with genes may be from damage to the gene or may be inherited. Damage to genes can come from many sources inside or outside of the cell. Faults in two types of genes are especially important: oncogenes, which drive the growth of cancer cells, and tumor suppressor genes, which prevent cancer from developing.

2.1.1 Causes

Determining what causes cancer is complex and it is often impossible to assign a specific cause for a specific cancer. Many things are known to increase the risk of cancer including tobacco use, infection, radiation, lack of physical activity, poor diet and obesity, and environmental pollutants (Anand et *al.*, 2008). These can directly damage genes or combine with existing genetic faults within cells to cause the disease (Kinzler *et al.*, 2002). A small percentage of cancers, approximately five to ten percent, are entirely hereditary.

Cancer can be detected in a number of ways, including the presence of certain signs and symptoms, screening tests, or medical imaging. Once a possible cancer is detected it is diagnosed by microscopic examination of a tissue sample. Cancer is usually treated with chemotherapy, radiation therapy and surgery. The chances of surviving the disease vary greatly by the type and location of the cancer and the extent of disease at the start of treatment. While cancer can affect people of all ages, and a few types of cancer are more common in children. The risk of developing cancer generally

increases with age. In 2007, cancer caused about 13% of all human deaths worldwide (7.9 million). Rates are rising as more people live to an old age and as mass lifestyle changes occur in the developing world (Jemal *et al.*, 2011).

Cancers are primarily an environmental disease with 90-95% of cases attributed to environmental factors and 5-10% due to genetics (Anand *et al.*, 2008). Environmental, as used by cancer researchers, means any cause that is not genetic, not merely pollution. Common environmental factors that contribute to cancer death include tobacco (25-30%), diet and obesity (30-35%), infections (15-20%), radiation (both ionizing and non-ionizing, up to 10%), stress, lack of physical activity, and environmental pollutants (Anand *et al.*, 2008). Cancer pathogenesis is traceable back to DNA mutations that impact cell growth and metastasis. Substances that cause DNA mutations are known as mutagens, and mutagens that cause cancers are known as carcinogens. Many mutagens are also carcinogens, but some carcinogens are not mutagens. Alcohol is an example of a chemical carcinogen that is not a mutagen (Seitz *et al.*, 1998). In Western Europe 10% of cancers in males and 3% of cancers in females are attributed to alcohol (Schutze, 2011).

Decades of research has demonstrated the link between tobacco use and cancer in the lung, larynx, head, neck, stomach, bladder, kidney, esophagus and pancreas (Kuper *et al.*, 2002). Tobacco smoke contains over fifty known carcinogens, including nitrosamines and polycyclic aromatic hydrocarbons (Kuper *et al.*, 2002). Tobacco is responsible for about one in three of all cancer deaths in the developed world (Sasco *et al.*, 2004) and about one in five worldwide (Kuper *et al.*, 2002). Diet, physical inactivity, and obesity are related to approximately 30–35% of cancer cases (Kushi *et al.*, 2006 and Anand *et al.*, 2008). Diets that are low in vegetables, fruits and whole grains, and high in processed or red meats are linked with a number of cancers (Kushi *et al.*, 2006). Worldwide approximately 18% of cancers are related to infectious diseases (Anand *et al.*, 2008). Viruses are usual infectious agents that cause cancer but bacteria and parasites may also have an effect. A virus that can cause cancer is called an oncovirus. Up to 10% of invasive cancers are

related to radiation exposure, including both ionizing radiation and non-ionizing radiation (Anand *et al.,* 2008).

The vast majority of cancers are non-hereditary, which are called sporadic cancers. Hereditary cancers are cancers that are primarily caused by an inherited genetic defect. Less than 0.3% of the population are carriers of a genetic mutation which has a large effect on cancer risk. They cause less than 3–10% of all cancers (Roukos, 2009).

Some substances cause cancer primarily through their physical, rather than chemical, effects on cells. A prominent example of this is prolonged exposure to asbestos, naturally occurring mineral fibers which are a major cause of mesothelioma, a type of lung cancer (Maltoni *et al.,* 2000). Physical trauma resulting in cancer is relatively rare. One accepted source is frequent, long-term application of hot objects to the body. It is possible that repeated burns on the same part of the body, such as those produced by kanger and kairo heaters may produce skin cancer, especially if carcinogenic chemicals are also present (Gaeta, 2000). Frequently drinking scalding hot tea may produce esophageal cancer (Gaeta, 2000).

Cancer is fundamentally a disease of failure of regulation of tissue growth. In order for a normal cell to transform into a cancer cell, the genes which regulate cell growth and differentiation must be altered (Croce, 2008). The affected genes are divided into two broad categories. Oncogenes are genes which promote cell growth and reproduction. Tumor suppressor genes are genes which inhibit cell division and survival. Malignant transformation can occur through the formation of novel oncogenes, the inappropriate over-expression of normal oncogenes, or by the under-expression or disabling of tumor suppressor genes. Typically, changes in many genes are required to transform a normal cell into a cancer cell (Knudson 2001).

Cancer has a reputation as a deadly disease. In 2008 approximately 12.7 million cancers were diagnosed (excluding non-melanoma skin cancers and other non-invasive cancers) and 7.6 million people died of cancer worldwide (Jemal *et al.,* 2011). Cancers as a group account for approximately 13% of all deaths each year with the most common being: lung cancer (1.3

million deaths), stomach cancer (803,000 deaths), colorectal cancer (639,000 deaths), liver cancer (610,000 deaths), and breast cancer (519,000 deaths) (WHO, 2006). This makes invasive cancer the leading cause of death in the developed world and the second leading cause of death in the developing world (Jemal *et al.*, 2011). Over half of cases occur in the developing world (Jemal, 2011). Cancer is regarded as a disease that must be "fought" to end.

2.2 Colorectal cancer

Colorectal cancer, commonly known as bowel cancer, is a cancer caused by uncontrolled cell growth (neoplasia) in the colon, rectum, or vermiform appendix. Colorectal cancer is clinically distinct from anal cancer, which affects the anus. Colorectal cancers start in the lining of the bowel. If left untreated, it can grow into the muscle layers underneath, and then through the bowel wall. Most begin as a small growth on the bowel wall: a colorectal polyp or adenoma. These mushroom-shaped growths are usually benign, but some develop into cancer over time. Localized bowel cancer is usually diagnosed through colonoscopy. Invasive cancers that are confined within the wall of the colon (TNM stages I and II) are often curable with surgery. However, if left untreated, the cancer can spread to regional lymph nodes (stage III). Cancer that has spread widely around the body (stage IV) is usually not curable. Colorectal cancer is the third most commonly diagnosed cancer in the world, but it is more common in developed countries (http://esa.un.org). Around 60% of cases were diagnosed in the developed world (http://globocan.iarc.fr/).

2.2.1 Symptoms

The symptoms of colorectal cancer depend on the location of tumor in the bowel, and whether it has spread elsewhere in the body (metastasis). While no symptom is diagnostic of colorectal cancer, rectal bleeding or anemia are high risk features (Astin *et al.*, 2011). There may be a change in bowel habit (such as unusual and unexplained constipation or diarrhea), and a feeling of incomplete defecation (rectal tenesmus). Lower gastrointestinal bleeding, including the passage of bright red blood in the stool, may indicate colorectal cancer, as may the increased presence of mucus. Melena, black stool with a

tarry appearance, normally occurs in upper gastrointestinal bleeding (such as from a duodenal ulcer), but is sometimes encountered in colorectal cancer when the disease is located in the beginning of the large bowel. A tumor that is large enough to fill the entire lumen of the bowel may cause bowel obstruction. This situation is characterized by constipation, abdominal pain, abdominal distension and vomiting. This occasionally leads to the obstructed and distended bowel perforating and causing peritonitis. A large left colonic tumor may compress the left ureter and cause hydronephrosis. Colorectal cancer may also lead to weight loss, generally due to appetite. There may be rarer symptoms including unexplained fever or thrombosis, usually deep vein thrombosis. Such symptoms, known as paraneoplastic syndrome, are due to the body's immune response to the cancer, rather than the tumor itself. The lifetime risk of developing colon cancer in the United States is about 7%. Certain factors increase a person's risk of developing the disease (Levin and Dozois, 1991). These include: age, polyps of the colon, history of cancer, heredity, smoking, physical inactivity, inflammatory bowel disease and environmental factors.

2.2.2 Origin

Colorectal cancer is a disease originating from the epithelial cells lining the colon or rectum of the gastrointestinal tract, most frequently as a result of mutations in the Wnt signaling pathway that artificially increase signaling activity. The mutations can be inherited or are acquired, and must probably occur in the intestinal crypt stem cell (Ionov *et al.,* 1993 and Srikumar *et al.,* 1999). The most commonly mutated gene in all colorectal cancer is the *APC* gene, which produces the APC protein. The APC protein is a "brake" on the accumulation of β-catenin protein; without APC, β-catenin accumulates to high levels and translocates (moves) into the nucleus, binds to DNA, and activates the transcription of genes that are normally important for stem cell renewal and differentiation but when inappropriately expressed at high levels can cause cancer. While APC is mutated in most colon cancers, some cancers have increased β-catenin because of mutations in β-catenin (*CTNNB1*) that block its degradation, or they have mutation(s) or other genes with function analogous to APC such as *AXIN1*, *AXIN2*, *TCF7L2*, or the Naked cuticle

(Nkd) gene *NKD1*(Markowitz and Bertagnolli, 2009). Beyond the defects in the Wnt-APC-beta-catenin signaling pathway, other mutations must occur for the cell to become cancerous. The p53 protein, produced by the *TP53* gene, normally monitors cell division and kills cells if they have Wnt pathway defects. Eventually, a cell line acquires a mutation in the *TP53* gene and transforms the tissue from an adenoma into an invasive carcinoma (Markowitz and Bertagnolli, 2009).

Some genes are oncogenes - they are over expressed in colorectal cancer. For example, genes encoding the proteins KRAS, RAF and PI3K, which normally stimulate the cell to divide in response to growth factors, can acquire mutations that result in over-activation of cell proliferation(Vogelstein and Kinzler, 2004).

2.2.3 Detection

Colorectal cancer can take many years to develop and early detection of colorectal cancer greatly improves the chances of a cure. Therefore, screening for the disease is recommended in individuals who are at increased risk. There are several different tests available for this purpose.

- Digital rectal exam (DRE): The doctor inserts a lubricated, gloved finger into the rectum to feel for abnormal areas. It only detects tumors large enough to be felt in the distal part of the rectum but is useful as an initial screening test.
- Fecal occult blood test (FOBT): A test for blood in the stool. Two types of tests can be used for detecting occult blood in stools i.e. guaiac based (chemical test) and immunochemical. The sensitivity of immunochemical testing is superior to that of chemical testing without an unacceptable reduction in specificity (Weitzel, 1999).
- M2-PK: A CE marked stool test which indicates colorectal polyps, colorectal cancer, acute and chronic inflammatory bowel disease and other diseases of the digestive tract. The test result is not affected by any foods, so no dietary restrictions are necessary before taking the stool sample. It detects bleeding and non-bleeding colorectal polyps and tumors and has significantly superior sensitivity compared to conventional occult blood

tests (Koss *et al.*, 2008). The amount of M2-PK in stool can be quantified in 4 mg of feces either by ELISA or with a Point-of-Care Rapid Test.

- Endoscopy:

  Sigmoidoscopy: A lighted probe (Sigmoidoscope) is inserted into the rectum and lower colon to check for polyps and other abnormalities.

  Colonoscopy: A lighted probe called a Colonoscope is inserted into the rectum and the entire colon to look for polyps and other abnormalities that may be caused by cancer. A colonoscopy has the advantage that if polyps are found during the procedure they can be removed immediately. Tissue can also be taken for biopsy.

- Blood tests: Measurement of the patient's blood for elevated levels of certain proteins can give an indication of tumor load. In particular, high levels of carcinoembryonic antigen (CEA) in the blood can indicate metastasis of adenocarcinoma. These tests are frequently false positive or false negative, and are not recommended for screening, it can be useful to assess disease recurrence.

2.2.4 Pathology

The pathology of the tumor is usually reported from the analysis of tissue taken from a biopsy or surgery. A pathology report will usually contain a description of cell type and grade. The most common colon cancer cell type is adenocarcinoma which accounts for 95% of cases. Other, rarer types include lymphoma and squamous cell carcinoma.

2.2.5 Staging

Colon cancer staging is an estimate of the amount of penetration of a particular cancer. The systems for staging colorectal cancers depend on the extent of local invasion, the degree of lymph node involvement and whether there is distant metastasis. Definitive staging can only be done after surgery has been performed and pathology reports reviewed. An exception to this principle would be after a colonoscopic polypectomy of a malignant pedunculated polyp with minimal invasion. Preoperative staging of rectal cancers may be done with endoscopic ultrasound. The most common staging system is the TNM (for tumors/nodes/metastases) system, from the American Joint Committee on Cancer (AJCC staging manual, 2002). The TNM system assigns a number

based on three categories. "T" denotes the degree of invasion of the intestinal wall, "N" the degree of lymphatic node involvement, and "M" the degree of metastasis. The broader stage of a cancer is usually quoted as a number I, II, III, IV derived from the TNM value grouped by prognosis; a higher number indicates a more advanced cancer and likely a worse outcome. Details of this system are shown in table 1:

Table 1: TNM system of staging (AJCC staging manual, 2002)

AJCC stage	TNM stage	(AJCC Cancer Staging Manual, 2002)
Stage 0	Tis N0 M0	Tis: Tumor confined to mucosa; cancer-*in-situ*
Stage I	T1 N0 M0	T1: Tumor invades submucosa
Stage I	T2 N0 M0	T2: Tumor invades muscularis propria
Stage II-A	T3 N0 M0	T3: Tumor invades subserosa or beyond (without other organs involved)
Stage II-B	T4 N0 M0	T4: Tumor invades adjacent organs or perforates the visceral peritoneum
Stage III-A	T1-2 N1 M0	N1: Metastasis to 1 to 3 regional lymph nodes. T1 or T2.
Stage III-B	T3-4 N1 M0	N1: Metastasis to 1 to 3 regional lymph nodes. T3 or T4.
Stage III-C	any T, N2 M0	N2: Metastasis to 4 or more regional lymph nodes. Any T.
Stage IV	any T, any N, M1	M1: Distant metastases present. Any T, any N.

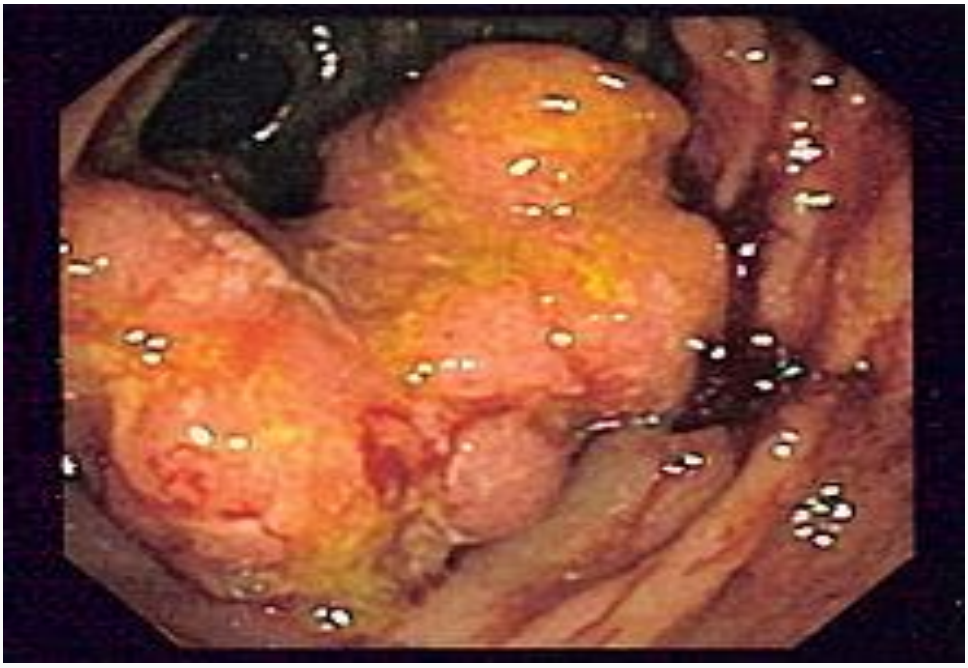

Fig.1: Endoscopic image of colon cancer identified in sigmoid colon on screening colonoscopy (Source: Wikipedia)

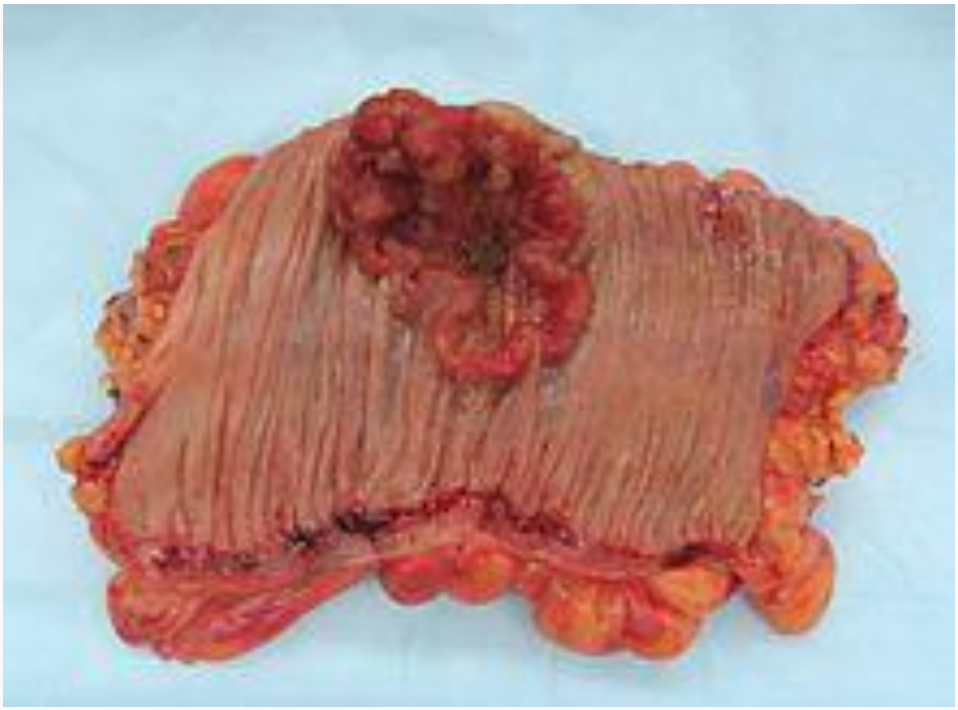

Fig.2: Gross appearance of a colectomy specimen containing invasive colorectal carcinoma (Source: Wikipedia)

2.2.5.1 Dukes system

The Dukes classification (Dukes, 1932) is an older and less complicated staging system, which predates the TNM system. It identified the stages as:

- A - Tumour confined to the intestinal wall
- B - Tumour invading through the intestinal wall
- C - With lymph node(s) involvement (this is further subdivided into C1 lymph node involvement where the apical node is not involved and C2 where the apical lymph node is involved)
- D - With distant metastasis

2.2.6 Incidence

The incidence of colorectal cancer varies greatly between different regions of the world, much of it can be attributed to differences in diet, particularly the consumption of red and processed meat, fibre and alcohol, as well as bodyweight and physical activity (Cancer Stats). Incidence rates of colorectal cancer are increasing in countries where rates were previously low (especially in Japan, but also in other Asian countries) as diets become more Westernized, and either gradually increasing, stabilising (Northern and Western Europe) or declining (North America) with time. In 2008, almost 60% of cases were diagnosed in the developed world (Cancer Stats). Colorectal cancer (CRC) is the third most common cause of cancer-related death in the western world. The annual incidence of CRC worldwide has been estimated to be at least half a million (Kemp *et al.,* 2004). It is a commonly diagnosed cancer in both men and women. In 2008, about 148,810 new cases were diagnosed, and almost 49,960 deaths from colorectal cancer were speculated (American Cancer Society, 2008). A major cause of death in CRC is the development of distant metastases through the spreading of tumor cells from the primary tumor site. Thus, it is important to find biological markers that can identify CRC patients that would benefit from adjuvant treatment due to an increased risk of recurrence. It would also be of great value to identify premalignant alterations in macroscopically normal-appearing colorectal mucosa that could be used in screening tests for patients at risk of developing CRC.

Kashmir valley located in the northern division of India, surrounded by Himalayas has a unique ethnic population living in a temperate environmental conditions having distinctive food habits which play an overwhelming role in the development of GIT cancers over the genetic factors (Shah and Jan, 1990; Murtaza *et al.,* 2006; Salam *et al.,* 2009). The food habits include consumption of sun-dried and smoked fish and meat, dried and pickled vegetables, red chili, Hakh (a leafy vegetable of Brassica family), hot noon chai (salted tea) and Hukka (water pipe) smoke (Mir *et al.,* 2005). As previously reported (Siddiqi *et al.,* 1992) the etiology and incidence of various GIT cancers in this population has been attributed to a probable exposure to nitroso compounds, amines and nitrates reported to be present in these local food stuffs. Colorectal cancer being the commonest cancer, is the major cause of mortality and morbidity worldwide, there are nearly one million new cases of colorectal cancer diagnosed world-wide each year and half a million deaths (Boyle and Elena, 2002). The incidence of this malignancy shows considerable variation among racially or ethnically defined populations in multiracial/ethnic countries. Kashmir has been reported by now as a high-incidence area of GIT cancers (Shah and Jan, 1990; Mir *et al.,* 2005; Salam *et al.,* 2009). Colorectal Cancer in Kashmir valley is the third most common GIT cancer after esophageal and gastric cancer.

2.2.7 Causes

Cancer formation is a multistep process in which defects in a wide range of cancer genes accumulate (Balmain *et al.,* 2003). Eventually every cancer receives an enormous complexity of altered gene functions, including activation of proto-oncogene as well as silencing of genes with tumor-suppressing function (Ilyas *et al.,* 1999). Genetic alternations including mutation, deletion, and DNA amplification have been shown to play an important role in tumorigenesis (Ingvarsson, 1999), however, the genetic abnormalities found in cancers will not provide the whole picture of genomic alternations.

The development of CRC is a multistep process, which can arise due to the cumulative effect of mutations in various different proto-oncogenes, tumor suppressor genes, and/or from epigenetic changes in DNA (Vogelstein *et al.,*

1988; Fearon *et al.*, 1990; Mustafa *et al.*, 2007). Recent progresses made in the field of molecular biology have shed light on the different alternative pathways involved in colorectal carcinogenesis, and more importantly on the cross talk among these pathways (Risques *et al.*, 2003; Takayama *et al.*, 2006).

2.2.8 Epigenetic mechanism

Epigenetic mechanisms are used in many different ways for control of gene expression. Epigenetic changes never involve a change in the primary DNA sequence or a change in base pairing but are reflected primarily in DNA cytosine modification patterns, histone post-translational modifications, or deposition of certain histone variants along specific gene sequences. These epigenetic modifications of genes are generally reversible, but can get transmitted to the daughter cells (Laird, 2005). For example, one type of epigenetic change that can occur is that the chromatin structure changes from an open active configuration, also referred to as euchromatin, to a densely packed inactive chromatin structure, the so-called heterochromatin. One common and perhaps the most permanent and stable mechanism of epigenetic gene inactivation is the methylation of the 5-carbon of the DNA base cytosine in the 5′-CpG-3′ dinucleotide sequence context of CpG island of promoter regions. These methylation reactions carried out by DNA cytosine methyltransferases are a main component of epigenetic regulatory mechanisms in mammals (Baylin *et al.*, 2001). Cytosine methylation of DNA at CpG dinucleotides is the most well known epigenetic change. Less than 10% of all cytosines are methylated in the human genome (Estellier, 2005). These CpG dinucleotides are also not found randomly throughout the genome, but in finite areas, specifically clustered at the 5' region of genes, in the promoter, untranslated region and exon 1. These CpG islands are not methylated in normal cells, thereby allowing for transcription of the genes through allowing access of the transcription machinery to the DNA. However, in cancer cells, these CpG dinucleotides found within these CpG islands is targeted and hypermethylated, thereby preventing any transcription activators to access the DNA. This is known as silencing, and often occurs on tumour-suppressor genes leading to tumorigenesis. Tumor-suppressor genes have an open chromatin conformation, with promoter CpG island hypomethylation. CpG

island hypermethylation closes the chromatin conformation through condensation and packaging, thereby allowing the cell entry in the cycle without regulation, and avoidance of cell death. Interestingly, in tumor cells, gene-specific 5' region CpG's are highly methylated, while the rest of the genome becomes highly unmethylated. This may be due to repetitive sequences and genome stability, and tumor cells exhibit genome fragility and chromatin instability.

Tumor suppressor genes were initially hypothesized to be inactivated in cancer cells as a result of genetic defects of both alleles (i.e., the Knudson two-hit hypothesis). However, there is now evidence that epigenetic events, such as hypermethylation of cytosine–guanine (CpG) sites in regulatory regions (e.g., the promoter), may be a critical alternative mechanism of tumor suppressor gene inactivation. In tumor tissues, tumor suppressor genes are often inactivated epigenetically by methylation when compared with normal tissue. The DNA methylation events are often preceded by changes in chromatin structure and histone modifications.

Epigenetic alternations of the DNA such as methylation of CpG island in promoter region or histone modification donot alter sequence code. Instead, they participate in the regulation of gene expression that is now recognized as an additional method to be involved in tumorigenesis (Plass, 2002; Jaenisch and Bird, 2003; Nephew and Huang, 2003). Methylation of cytosine residue at CpG dinucleotides in mammalian genomes is found to have significant effect on gene expression (Baylin and Herman, 2000; Ehrlich, 2002). In higher order eukaryotes, DNA is methylated only at cytosines located 5' to guanosine in the CpG dinucleotide (Holliday and Grigg, 1993). This modification has important regulatory effects on gene expression, especially when involving CpG-rich areas known as CpG islands, located in the promoter regions of many genes (Bird, 1986; 1992). While almost all gene-associated islands are protected from methylation on autosomal chromosomes (Bird, 1986), extensive methylation of CpG islands has been associated with transcriptional inactivation of selected imprinted genes (Li *et al.,* 1993; Tremblay *et al.,* 1995) and genes on the inactive X-chromosome of females (Pfeifer *et al.,* 1989;Riggs *et al.,* 1992).

Aberrant methylation of normally unmethylated CpG islands has been documented as a relatively frequent event in immortalized and transformed cells (Antequera *et al.,* 1990) and has been associated with transcriptional inactivation of defined tumor suppressor genes in human cancers (Herman *et al.,* 1994; Merlo *et al.,* 1995; Graff *et al.,* 1995; Herman *et al.,* 1996). In this situation, promoter region hypermethylation stands as an alternative to coding region mutations in eliminating tumor suppressor gene function (Herman *et al.,* 1994, Merlo *et al.,* 1995). Therefore, mapping of methylation patterns in CpG islands has become an important tool for understanding both normal and pathologic gene expression events.

2.2.9 *P16* gene

P16 plays an important role in regulating the cell cycle, and mutations in *p16* increase the risk of developing a variety of cancers, notably melanoma. The *p16* protein is encoded by the *CDKN2* gene. The *p16* (*CDKN2a/INK4a*) gene is an important tumor-suppressor gene, involved in the *p16*/cyclin-dependent kinase/retinoblastoma gene pathway of cell cycle control, in which the *p16* protein is considered to be a negative regulator involved in the inhibition of G1 phase progression (Rocco *et al.,* 2001). *P16* gene is located on human chromosome 9 with location 9P21 and is of length 8.5Kb. The complete *p16* memory is by two introns and three exons, coding a known cyclin-dependent kinase CDK4 inhibitor protein.

The molecular weight of it is 15.8kd and is referred to as *P16*.The human *p16* protein contains 156 amino acids and was first discovered in a yeast two-hybrid system to detect proteins that interact with human cyclin-dependent kinase (Ruas and Peters, 1998). The tumor-suppressor function of *p16* is attributed to its ability to inhibit the catalytic activity of the cyclin-dependent kinase 4-6/cyclin D complex that is required for phosphorylation of retinoblastoma protein (Serrano *et al.,* 1993; 1996). Dephosphorylated Rb protein-binding transcription factors (such as the EIF) can not be released activation. The cells get arrested in G0 phase or G1 phase, while inhibiting cell division and proliferation. *P16* gene abnormal changes lead to the loss of the negative regulation of cell growth. CDK4 and cyclin D1 binding to Rb protein lead to

its phosphorylation and in the release of a large number of transcription factors which change G1 phase cells into S phase cells rapidly.

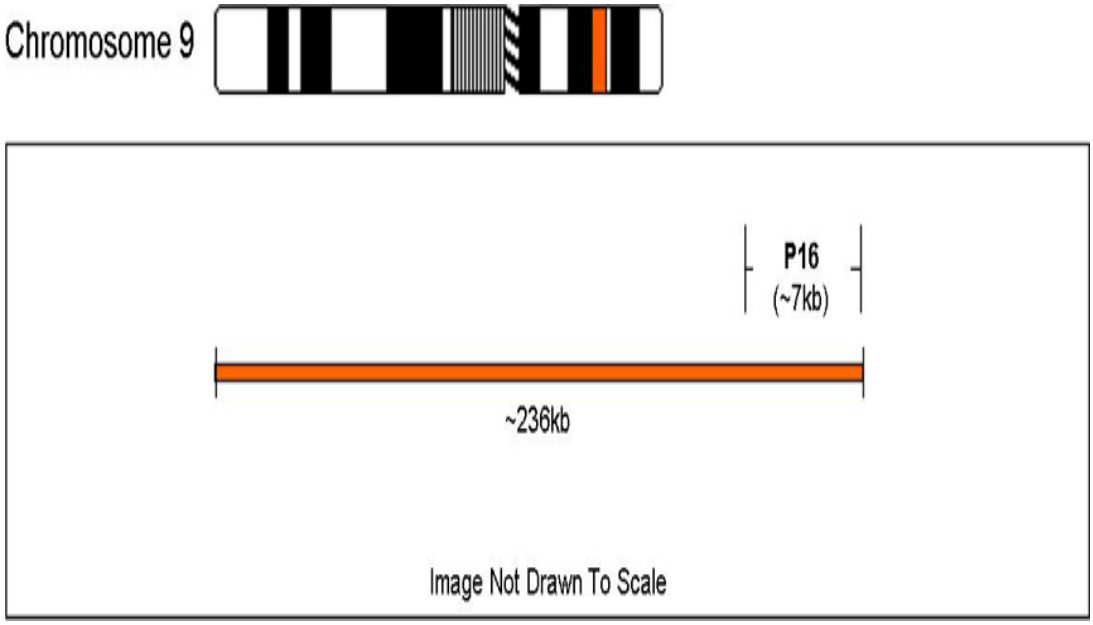

Fig.3: Chromosomal location of *P16* gene. (Source: Pubmed)

2.2.10 Inactivation of *p16* gene

Inactivation of *p16* gene plays an important role not only in tumorigenesis but is also considered to be a poor prognostic event in certain malignancies (Serrano *et al.*, 1993; Serrano *et al.*, 1996; Ruas and Peters, 1998; Rocco *et al.*, 2001). DNA methylation is a frequent mechanism of transcriptional silencing in human cancer (Herman *et al.*, 2000). Recently, aberrant methylation of multiple promoter-associated CpG islands of tumor-suppressor genes has been found in a variety of human malignancies. Promoter hypermethylation, in addition to gene deletion and point mutation of *p16* locus, has been found to be one of the main mechanisms of *p16* inactivation (Cairns *et al.*, 1995). Hypermethylation of the 5'CpG islands of *p16* gene promotor region has been reported in colon, bladder, breast, head and neck, and lung carcinoma as well as in glioma, melanoma, leukemia and lymphoma (Reznikoff *et al.*, 1996). However, there appears to be evidence supporting a role for *p16* inactivation in the transformation of low-growth fraction lymphomas into their aggressive variants (Navas *et al.*, 2002). The histological progression of follicular lymphoma to aggressive large-cell lymphoma is frequently accompanied by 9p21 deletions that often result in reduction or loss of *p16* expression. In mantle cell lymphoma, *p16* deletions are associated with aggressive forms, a finding that suggests the possibility that cyclin D1 overexpression and *p16* inactivation might not be completely redundant alterations (Sanchez-Beato *et al.*, 2003). Mechanisms of *p16* silencing include loss of heterozygosity, homozygous deletion, point mutation and promoter region hypermethylation. The latter appears to be a major reason for *p16* inactivation in many previous studies. There appears to be good agreement of *p16* promoter methylation with lack of protein expression in B-cell lymphomas (Sanchez-Beato *et al.*, 2001; 2003).

Hypermethylation of the *p16* tumour suppressor gene and subsequent transcriptional silencing has been implicated as an additional mechanism of *p16* gene inactivation in diverse types of cancer including gastric cancer, lung cancer, colon cancer, thyroid carcinoma, and hepatic carcinoma (Elisei *et al.*, 1998; Gazzeri *et al.*, 1998; Shim *et al.*, 2000; 2003; Ding *et al.*, 2003). Methylation of *p16* gene correlated with decrease expression in human gastric

cancer (Shim *et al.,* 2000) and hypermethylation of *p16* occurs in the early stage of hepatic cell carcinomas and is associated with hepatitis B virus infection (Shim *et al.,* 2003). Promoter hypermethylation of several tumor suppressor genes such as *p16*, *p15*, *E-cadherin* were also found in different type of leukemias (Gutierrez *et al.,* 2003; Galm *et al., 2004*). The relationship of *p16* inactivation or aberrant genetic or epigenetic changes of the *p16* gene with tumor progression or patient survival has been investigated in various forms of human tumor, such as breast cancer (Child *et al.,* 2002), cutaneous melanoma (Straume and Akslen, 1997), colorectal carcinoma (Esteller *et al.,* 2001), central nervous system malignancies (Straume *et al.,* 2000), lung cancer (Kim *et al.,* 2001) and head and neck carcinoma (Bova *et al.,* 1999) with no general consensus. However, before accepting the conclusion that hypermethylation of tumor suppressor gene promoters is invariably the cause of gene inactivation, it is worth evaluating the data a bit more critically. Epigenetic mechanisms of gene inactivation, including promoter hypermethylation, are undoubtedly important in cancer development and represent an alternative means of inactivating tumor suppressor genes. Nevertheless, the standard of proof for establishing that hypermethylation of the promoter of any given gene has a critical role in loss of gene expression and cancer development should probably be set quite high, regardless of whether the gene is a well-established tumor suppressor gene, like *p16*, or a potential tumor suppressor gene. Evidence might include data indicating that the methylation status of a promoter is tightly linked to its expression in a large panel of primary cancer specimens and data showing that gene expression can be readily and fully restored by treatment of cancer cells with demethylating agents, it is worth bearing in mind that, in some cases, promoter hypermethylation may be a reflection rather than a cause of gene inactivation in cancer. In this study, the aberrant promoter hypermethylation of *p16* gene in colorectal cancer was demonstrated. The result suggested that promoter methylation of this gene plays an important role in colorectal tumorigenesis.

Materials and Method:

The study was a hospital based case control study undertaken to understand the etiology of colorectal cancer in Kashmir valley-a north Indian state with high incidence rate of this dreadful disease. The research work was initiated following approval by the Board of Research Studies (BORS) of the University of Kashmir. All ethical considerations were taken care of during the study and the recruitment process was started only after ethical clearance from the Departmental Ethical Committee as per norms. Subjects with histopathologically and endoscopically confirmed colorectal carcinoma were evaluated. The colorectal carcinoma samples were collected from the Department of Surgery, Government S.M.H.S (Shri Maharaja Hari Singh) Hospital associated with Government Medical College, Srinagar either by means of surgical resection or endoscopically. Histopathologically confirmed normal were taken as controls. The study included 70 surgically obtained colorectal samples among which 50 were obtained from colorectal cancer patients and 20 were histopathologically normal colorectal samples. All the samples were histopathologically confirmed before further processing. Record was maintained of the complete case history of the patients. The various methods that were used to analyse the epigenetic silencing of tumour suppressor gene *p16* by promoter hypermethylation at CpG islands as a part of this study are described under:

3.1. CASES

All the colorectal cancer patients that were 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.

3.1.1. Inclusion criteria

The diagnosis of colorectal 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, gender.
- Native patients belonging to Kashmir valley.

3.1.2. 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 with genetic disorders like HNPCC/ APC.
- Patients who suffered from any other kind of malignancy

3.2. CONTROLS

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

3.2.1. Inclusion criteria

- Native populace of the Kashmir valley.

3.2.2. Exclusion criteria

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

3.3. COLLECTION OF TISSUE SAMPLES

The carcinoma and control samples were obtained from the Department of Surgery and Endoscopic Section of Government S.M.H.S Hospital and were put in sterilized 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. The vials were properly labelled according to a specially designed coding system. This coding system was developed so as to prevent possible mixing of the sample vials and for easy retrieval of the required sample vial. Also, a part of each sample was sent to histopathology laboratory of S.M.H.S hospital for histopathological confirmation. The information regarding the gender, stage and histological grade for each carcinoma sample was collected from the histopathological reports. The information regarding the gender of control samples was also collected.

3.4. GENETIC ANALYSIS

3.4.1. Extraction of genomic DNA

For the isolation of genomic DNA, kit based method was used. The kit used was Quick- g DNA™ MiniPrep supplied by ZYMO RESEARCH. The protocol followed was as per kit.

- 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 lyaste was centrifuged at 10,000 X g for 5 min. and the supernatant transferred to Zymo-Spin™ 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™ column transferred to a new collection tube and 200 µl of DNA pre-wash buffer (provided in the kit) 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™ 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.

3.4.2. QUALITATIVE AND QUANTITATIVE ANALYSIS OF GENOMIC DNA

3.4.2.1. Qualitative Analysis

The integrity of the genomic DNA was examined by gel electrophoresis using 1 % agarose gel. 2µl of each DNA sample was mixed with 1µl of 1X DNA loading dye (1X loading dye consists of 4.16 mg bromophenol blue, 4.16 mg xylene cyanol and 0.66g sucrose in1ml water) and was loaded in the gel. Electric current was applied at 50 volt until DNA entered in to the gel and was increased to 70 volt for rest of the run. Electrophoresis 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.

3.4.2.2. Quantitative Analysis

The quantity of the DNA was determined by measuring optical density at 260nm and 280 nm by double beam spectrophotometer (Evolution 60S from Thermo Scientific) and the concentration was determined by using the fact that absorbance of 1 unit equates to 50µg of DNA/ cm^3 and therefore, the concentration of DNA sample is given by the following equation

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

The ratio of 260/280nm was calculated and the DNA samples for which the ratio was 1.7-1.9 were considered for the future use. DNA was alliqouted into three to four tubes so as to protect damage from freeze thawing and stored in -20^{o}C freezer for longer duration of time.

3.5. DNA MODIFICATION (BISULFITE TREATMENT)

Sodium bisulfite treatment converted unmethylated cytosines to uracil and hence enabled to distinguish between the hypermethylated and non hypermethylated cytosine residues. DNA was modified by kit based method, the kit used was EZ DNA Methylation^{TM} Kit (The kit has > 99% conversion efficiency of converting non-methylated C residues into U, and the modified DNA recovery from the kit is > 80%). The protocol supplied with the kit was followed.

- 500-1000 ng of above isolated DNA samples were taken in different eppendorf tubes.
- To each tube 5 µl of M-Dilution Buffer provided in the kit was added and final volume made to 50 µl with distilled water.
- Then to each tube 100 µl CT-conversion reagent provided in the kit was added and all the tubes were kept in dark at 50^{o}C-55^{o}C for 12-16 hrs.
- After that all samples were placed on ice for 5-10 mins.

- 400 µl of M-binding buffer provided in the kit was added to zymo-spin™ IC columns provided in the kit and and the columns were placed into provided Collection Tubes.
- Sample (from step 4) were loaded into zymo-spin™ IC columns which contained M-binding buffer. The caps were closed and the solution mixed by inverting columns several times.
- Then columns were centrifuged at full speed (≥10,000× g) for 30 seconds and the flow-through discarded.
- 100 µl of M-wash buffer provided in the kit was added to these columns and then centrifuged at full speed for 30 seconds and the flow-through again discarded.
- 200 µl of M-desulphonation buffer provided in the kit was added to each column and then kept at room temperature (20^0C-30^0C) for 15-20 minutes. After the incubation, the columns were centrifuged at full speed for 30 seconds and the flow-through discarded.
- 200 µl of M-wash buffer was added to each column and then centrifuged at full speed for 30 seconds. Again 200 µl of M-wash buffer was added to each column and then centrifuged for an additional 30 seconds. The flow-through discarded each time.
- The columns were placed into 1.5 ml micro centrifuge tubes and 15 µl of M-elution buffer provided in the kit was directly added to each column matrix. Then these columns were centrifuged for 30 seconds at full speed and the DNA eluted.

The modified DNA was stored at -20^0C for further use.

3.6. METHYL SPECFIC POLYMERASE CHAIN REACTION (MSP)

To determine the status of *p16* promoter methylation in colorectal cases from Kashmir valley, we performed Methyl specific PCR (MSP) for the promoter region of *p16* gene in 50 surgically resected colorectal cancer DNA and compared with that of 20 histopathologically confirmed normal colorectal tissues. MSP is a novel and sensitive way for detection of hypermethylation in CpG islands of DNA (Herman et al., 1996). The sensitivity of MSP suggests it

would be useful for primary tumours as well, allowing for detection of aberrantly methylated alleles even if they contribute relatively little to the overall DNA in a sample.

The principle of this PCR method lies in the amplification of the hypermethylated and non-methylated DNA of the same gene by different primer sequence; one for hypermethylated version of the gene and one for the non-methylated version of the same gene. Thus by visualising the PCR product we can easily determine whether amplification is by methylated or non-methylated primers, thus determine whether CpG's were hypermethylated or non hypermethylated.

Primers described previously by Herman *et al*., 1996 were used to discriminate between methylated and unmethylated DNA following bisulfite treatment and to discriminate between DNA modified by bisulfite and that which had not been modified. To accomplish this, primer sequences has been chosen for regions containing frequent cytosines (to distinguish unmodified from modified DNA), and CpG pairs near the 3' end of the primers (to provide maximal discrimination in the PCR between methylated and unmethylated DNA). The fragment of DNA to be amplified was intentionally small, to allow the assessment of methylation patterns in a limited region and to facilitate the application of this technique to samples where amplification of larger fragments is not possible. In Table 2, primer sequences and amplicon size are shown for gene tested, emphasizing the differences in sequence between the types of DNA that are exploited for the specificity of MSP. The multiple mismatches in these primers, which are specific for these different types of DNA, suggest that each primer set should provide amplification only from the intended template.

Table 2: Primers (described by Herman et al, 1996) used and length of fragments obtained in MSP (Methylation Specific PCR)

Nature of Primer		Primer sequence	Size of Amplicon
UNMETHYLATED PRIMER	Forward primer	5′-TTATTAGAGGGTGGGGTGGATTGT-3′	151bp
	Reverse primer	5′-CAACCCCAAACCACAACCATAA-3′	
METHYLATED PRIMER	Forward primer	5′-TTATTAGAGGGTGGGGCGGATCGC-3′	150bp
	Reverse primer	5′-GACCCCGAACCGCGACCGTAA-3′	

As described earlier PCR was carried out using methyl specific PCR (MSP) method. The modified DNA was taken into two PCR vials in equal quantity and same amount of all reagents (Table 3) was added to both the vials except in one vial where methylated primers were added and in other vial where non-methylated primers were added.

PCR amplification was achieved using a Thermal cycler (Gradient thermal cycler from EPPENDORF MASTERCYCLER PRO). Reactions were hot-started at 95^{o}C for 5 min, followed by addition of Taq. Polymerase, followed by 35 cycles of melting (95^{o}C for 30 sec.), annealing (60^{0}C/65^{0}C for 30 sec.) and extension (72^{o}C for 30 sec.) and by final extension step at 72^{o}C for 4 min (Table 4). The annealing temperatures for unmethylated and methylated *p16* amplification was 60^{0}C and 65^{0}C, respectively.

Controls without DNA were performed for each set of PCRs. Universal Methylated Human DNA Standard and Control with primers was used as positive control, and Lymphocyte DNA was used as negative control.

After amplification each PCR product (10 μl) was directly loaded onto non denaturing 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

The amplified DNA were of different base pairs in length the methylated band was 150 bp and the unmethylated band was 151 bp and this small difference in the size of the amplified DNA did not allowed them to be visually differentiated in presence of a 100 bp DNA ladder run parallel to the amplified PCR products on 2% ethidium bormide pre-loaded agarose gel.

Table 3: Volume and concentrations of different reagents used in PCR

Reagent	Volume
1 X *Taq* buffer	2.5 µl
dNTPs (1.25mM/ L)	1.25 µl
Forward primer (150 ng/ reaction)	1µl
Reverse primer (150 ng/ reaction)	1 µl
Template DNA(50 ng/ reaction)	1.25 µl
Taq DNA Polymerase(5U/ µl)	0.2 µl
De ionised water	17.8 µl

Table 4: Thermal cycling conditions

Note: The annealing temperatures for unmethylated and methylated *p16* amplification was 60^0C and 65^0C, respectively.

Steps	Temperature ^{0}C	Time	Number of cycles
1. Hot-Start	95	5 min	1
2. Denaturation	95	30 sec	
3. Annealing	60/65	30 sec	35
4. Extension	72	30 sec	
5. Final extension	72	4 min	1

3.7. STATISTICAL ANALYSIS

The χ^2-test with Odds ratio was used to examine the differences in the distribution of *p16* gene promoter hypermethylation and non hypermethylation 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:

4.1.1. Cases

50 histopathologically confirmed cancer cases were taken. Out of 50 cases 29 were males which correspond to 58% and hence 21 cases out of 50 were females which correspond to 42%. All the patients were symptomatic at the time of diagnosis. Of all the ten districts highest number of colorectal cases turned out from Srinagar and Budgam districts of Kashmir.

4.1.2. Controls

20 histopathologically confirmed normal colorectal tissues were taken as controls. Out of 20 normal cases 10 were males which correspond to 50% and hence 10 cases out of 20 were females which correspond to 50%.

4.1.3. Extraction of genomic DNA

Genomic DNA was isolated by kit based method. The kit used was Quick- g DNA^{TM} MiniPrep supplied by ZYMO RESEARCH. Genomic DNA was successfully isolated from all 70(50 cases and 20 controls) tissue samples.

4.1.4. Qualitative analysis

The integrity of the genomic DNA isolated from tissue samples was examined on 1 % agarose gel. The representative gel picture of the isolated genomic DNA is given in the figure 4.

4.1.5. Quantitative Analysis

The quantity of the DNA was determined by measuring optical density at 260nm and 280 nm by double beam spectrophotometer (Evolution 60S from Thermo Scientific) and the concentration was determined using equation

$$\text{DNA } (\mu g/ml) = A_{260} \times 50 \times \text{dilution factor}$$

All 70 tissue samples (50 cases and 20 controls) included in the study were found to have the ratio of 260/280nm in the range of 1.7-1.9.

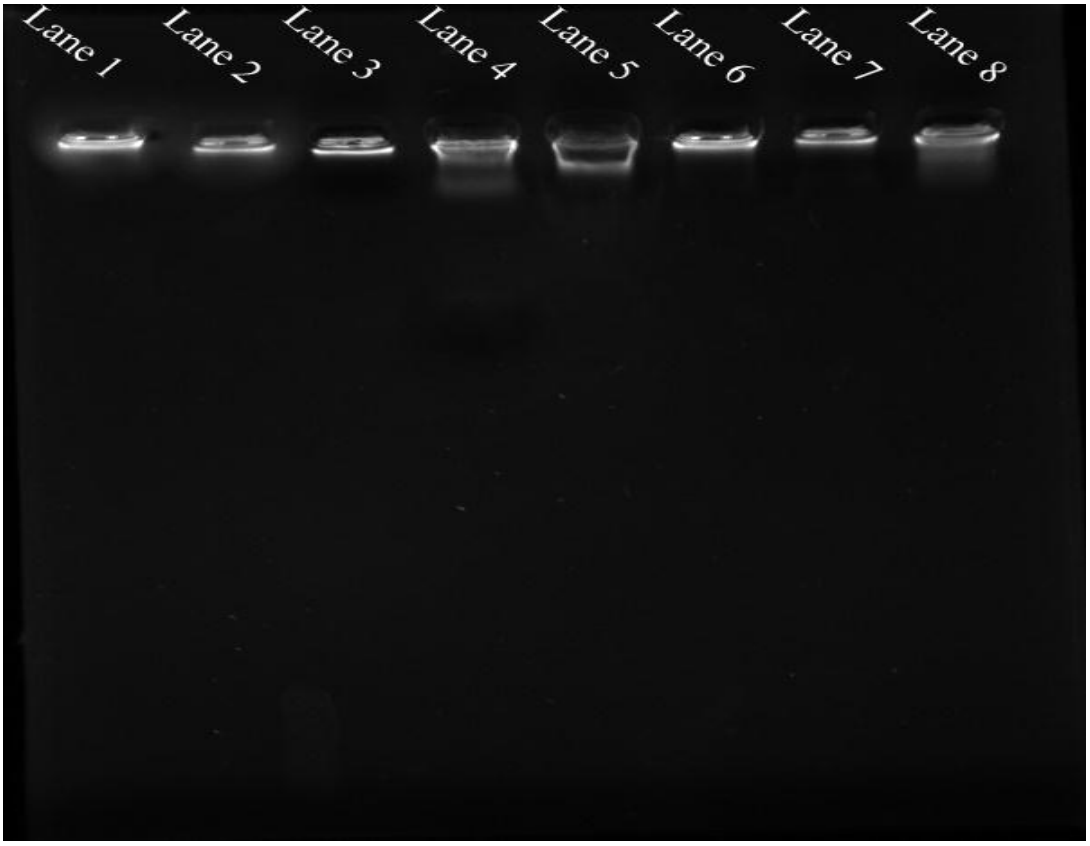

Fig.4: Representative gel picture showing the integrity of the genomic DNA on 1.0 % agarose gel.
Lane 1 to 8 contains the genomic DNA isolated from the tissue samples of colorectal cancer cases CRC 1 to CRC 8.

4.2. ANALYSIS OF *P16* GENE PROMOTER HYPERMETHYLATION IN CASES AND CONTROLS

To determine the status of *p16* promoter methylation in colorectal cases from Kashmir valley, we performed Methyl specific PCR (MSP) for the promoter region of *p16* gene in 50 surgically resected colorectal cancer DNA and compared with that of 20 histopathologically confirmed normal colorectal tissues. Primers described previously by Herman *et al*., 1996 were used to discriminate between methylated and unmethylated DNA following bisulfite treatment and to discriminate between DNA modified by bisulfite and that which had not been modified. The amplicons were analysed on 2% agarose gel. Amplification was carried out using Hot Start PCR method; the method involves heating the PCR mixture without Taq Polymerase upto 95^{o}C for 5 min. and then adding Taq Polymerase to it. This decreases the non specific amplifications.

As shown in Table 5 and Fig. 5, 66% (33/50) of the colorectal cancer tissues showed methylated *p16* promoter and 34% (17/50) of the cases however showed unmethylated *p16* promoter. Fig. 6 represented few cases among which CRC 1 and CRC 4 were found to be methylated and CRC 8 was unmethylated. Similarly Fig. 7 represented few cases among which CRC 38 was methylated and CRC 23 was unmethylated. Among the methylated cases few cases also showed the presence of a band of unmethylated DNA as represented in Fig. 7 by CRC 13, that could be derived from unmethylated DNA of normal, adjoining mucosal cells and tumor cells as well as normal constituents in the stroma such as vascular endothelial cells, smooth muscles, fibroblasts and inflammatory cells. Almost all 80% (16/20) of the histopathologically confirmed normal tissues showed unmethylated *p16* promoter except only in four cases where *p16* promoter was found to be methylated (Table 6 and Fig. 5). Fig. 8 represented few cases of normal samples like N3, N9 and N17 which showed unmethylated *p16* promoter. The association of promoter hypermethylation with colorectal cancer was evaluated by χ^2 (Chi square) test with Odds ratio and was found to be significant (P=0.0006, Odds ratio=7.765, 95% C.I=2.242 to 26.90).

Table 5: Data representing no. of cases showing promoter hypermethylation and non-hypermethylation during MSP amplification in colorectal cancer cases confirmed by 2% agarose gel electrophoresis

	CASES (50)	
PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATED	33	66 % (33/50)
NON HYPERMETHYLATED	17	34 % (17/50)

Table 6: Data representing no. of cases showing promoter hypermethylation and non-hypermethylation during MSP amplification in histopathologically confirmed normal cases confirmed by 2% agarose gel electrophoresis

CONTROL (20)		
PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATED	4	20 % (4/20)
NON HYPERMETHYLATED	16	80% (16/20)

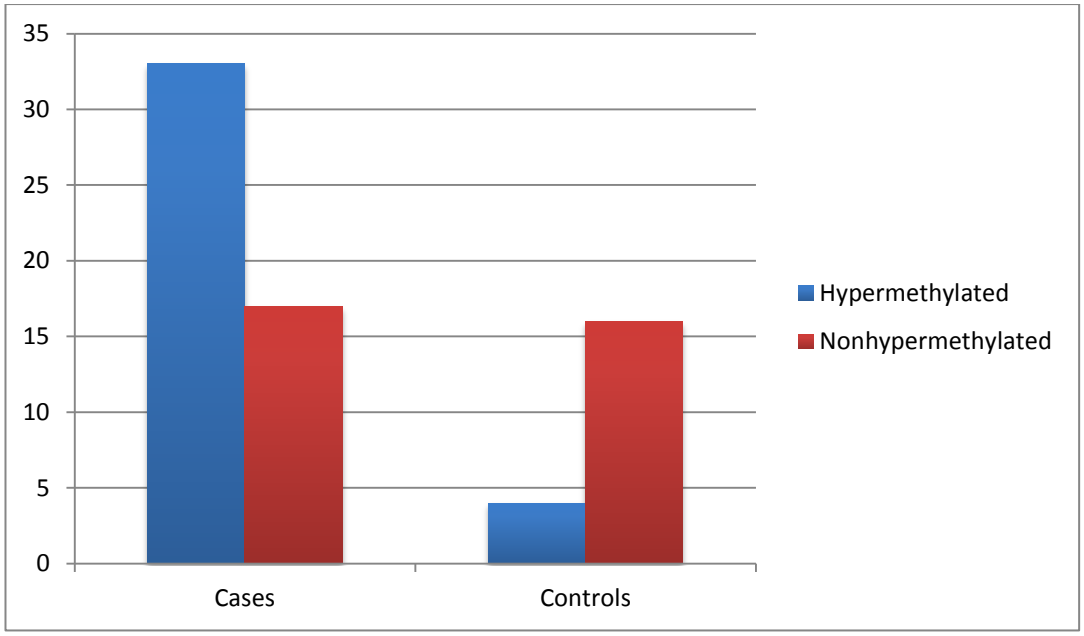

Fig.5: Histogram representing hypermethylated and nonhypermethylated cases of colorectal cancer and histopathologically confirmed normal controls

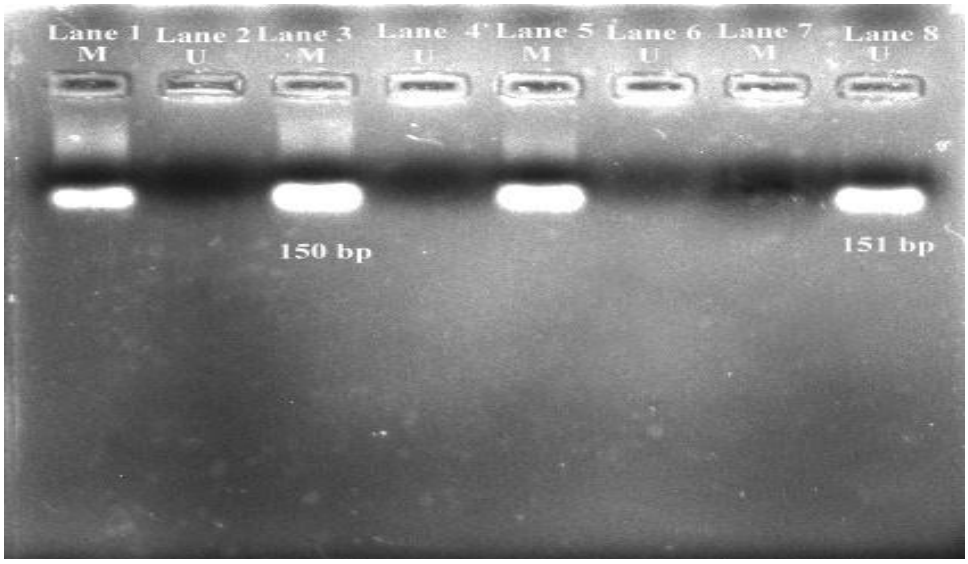

Figure 6. Representing MSP (Methylation Specific PCR) of colorectal cancer DNA samples run on 2% agarose gel

Lane 1 and 2-Represents positive control (universal methylated DNA) amplified with only methylated primer

Lane 3 and 4- Represents CRC1 amplified with only methylated primer

Lane 5 and 6- Represents CRC4 amplified with only methylated primer

Lane 7 and 8- Represents CRC8 amplified with only unmethylated primer

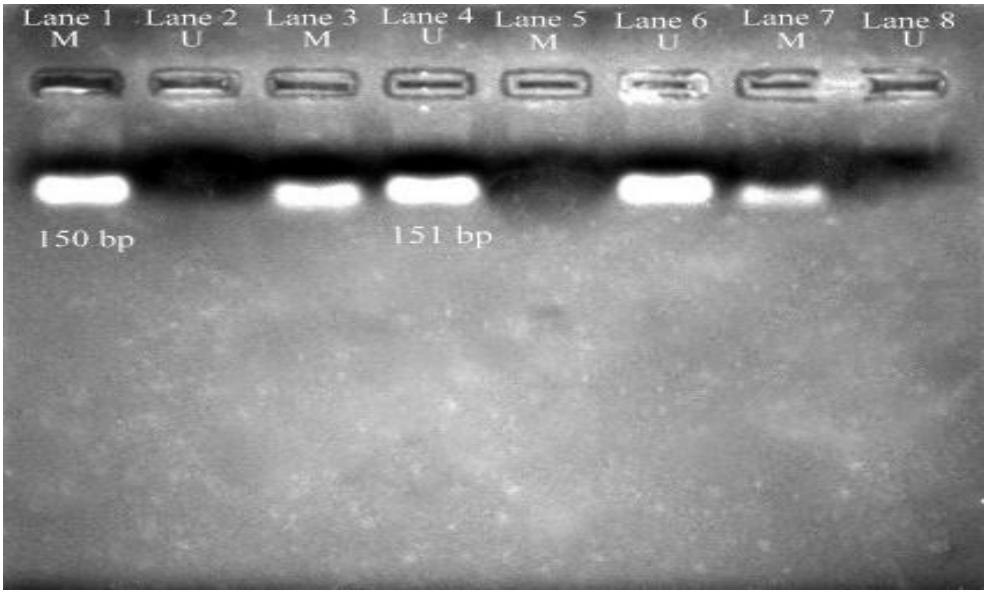

Figure 7. Representing MSP (Methylation Specific PCR) of colorectal cancer DNA samples run on 2% agarose gel

Lane 1 and 2-Represents positive control (universal methylated DNA) amplified with only methylated

Lane 3 and 4- Represents CRC13 amplified both with methylated primer and unmethylated primer

Lane 5 and 6- Represents CRC23 amplified with only unmethylated primer Lane 7 and 8- Represents CRC38 amplified with only methylated primer

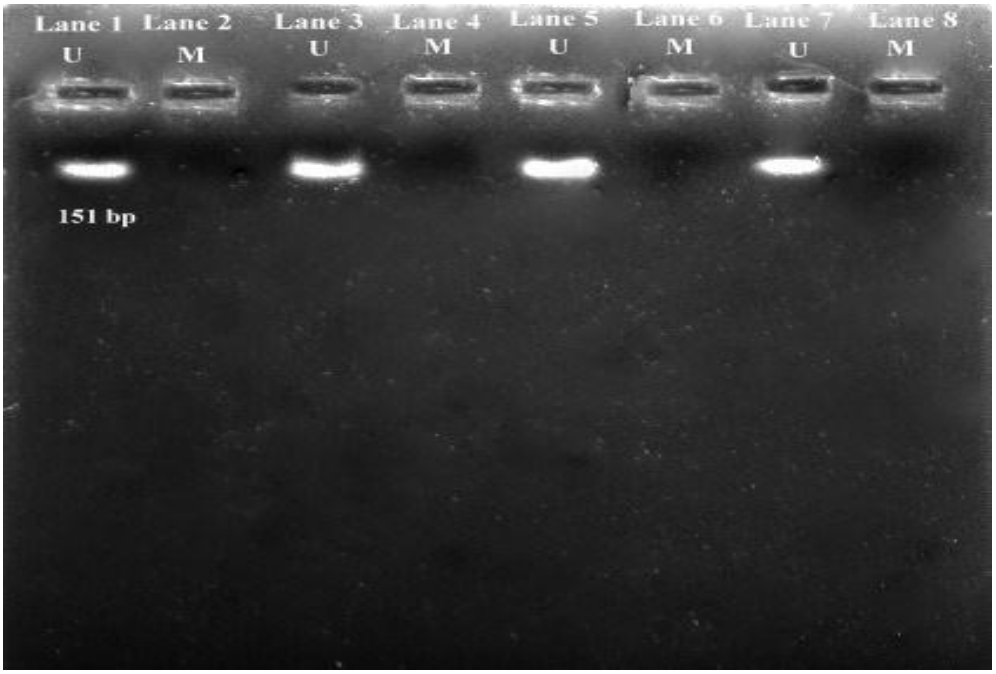

Figure 8. Representing MSP (Methylation Specific PCR) of histopathologically confirmed normal colorectal DNA samples run on 2% agarose gel

Lane 1 and 2-Represents negative control (lymphocyte DNA which is unmethylated) amplified with only unmethylated primer

Lane 3 and 4- Represents normal (N3) amplified with only unmethylated primer

Lane 5 and 6- Represents normal (N9) amplified with only unmethylated primer

Lane 7 and 8- Represents normal (N17) amplified with only unmethylated primer

4.3. Relationship between promoter hypermethylation of *p16* gene and selected clinicopathological parameters

The relationship between the promoter hypermethylation of *p16* gene and selected clinicopathological parameters was examined. These parameters included gender, clinical staging and histological grade.

4.3.1. Relationship of promoter hypermethylation of *p16* gene in males and females

Occurrence of *p16* methylation was found to be unequally distributed in males and females with more frequency in males than in females. Among 29 males, 20 cases were hypermethylated and 9 cases were unhypermethylated and among 10 male controls, 2 cases were hypermethylated and 8 cases were unhypermethylated (Table 7 and Fig. 9). The association of promoter hypermethylation with colorectal cancer was evaluated using Fisher's exact test and was found to be significant in males (P =0.0107, Odds ratio=8.889 and 95% C.I=1.563 to 50.55). In comparison, among 21 females, 13 cases were hypermethylated and 8 cases were unhypermethylated and among 10 female controls 1 case was hypermethylated and 9 cases were unhypermethylated (Table 8 and Fig.10). The association of promoter hypermethylation with colorectal cancer was again evaluated using Fisher's exact test and was found to be significant in females too (P =0.0089,Odds ratio=14.63 and 95% C.I=1.547 to 138.3). However, on comparing the male cases with female cases, 20 cases were hypermethylated and 9 cases were unhypermethylated in males and 13 cases were hypermethylated and 8 cases were unhypermethylated in females (Table 9 and Fig.11), occurrence of *p16* methylation was found to be unequally distributed in males and females with more frequency in males than in females but the difference was not statistically significant (*P* =0.7635, Odds ratio=1.368 and 95% C.I=0.4197 to 4.456).

Table 7: Data representing no. of cases showing promoter hypermethylation and non-hypermethylation in male colorectal cancer cases and male controls during MSP amplification confirmed by 2% agarose gel electrophoresis

MALE CASES (29)		
PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATED	20	68.97% (20/29)
NON HYPERMETHYLATED	9	31.03% (9/29)
MALES CONTROLS (10)		
PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATED	2	20 % (2/10)
NON HYPERMETHYLATED	8	80 % (2/10)

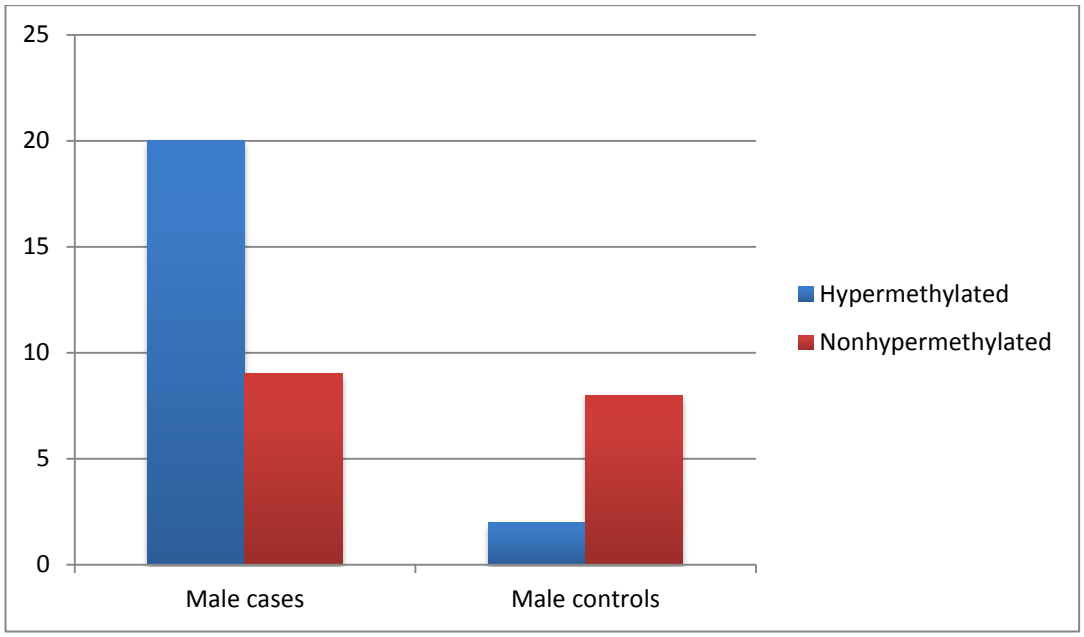

Fig.9: Histogram representing hypermethylated and nonhypermethylated male colorectal cancer cases and histopathologically confirmed normal male cases

Table 8: Representing no. of cases showing promoter hypermethylation and non-hypermethylation in female colorectal cancer cases and female controls during MSP amplification confirmed by 2% agarose gel electrophoresis

FEMALE CASES (21)		
PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATED	13	61.90 % (13/21)
NON HYPERMETHYLATED	8	38.09 % (8/21)
FEMALE CONTROLS (10)		
PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATED	1	10 % (1/10)
NON HYPERMETHYLATED	9	90 % (9/10)

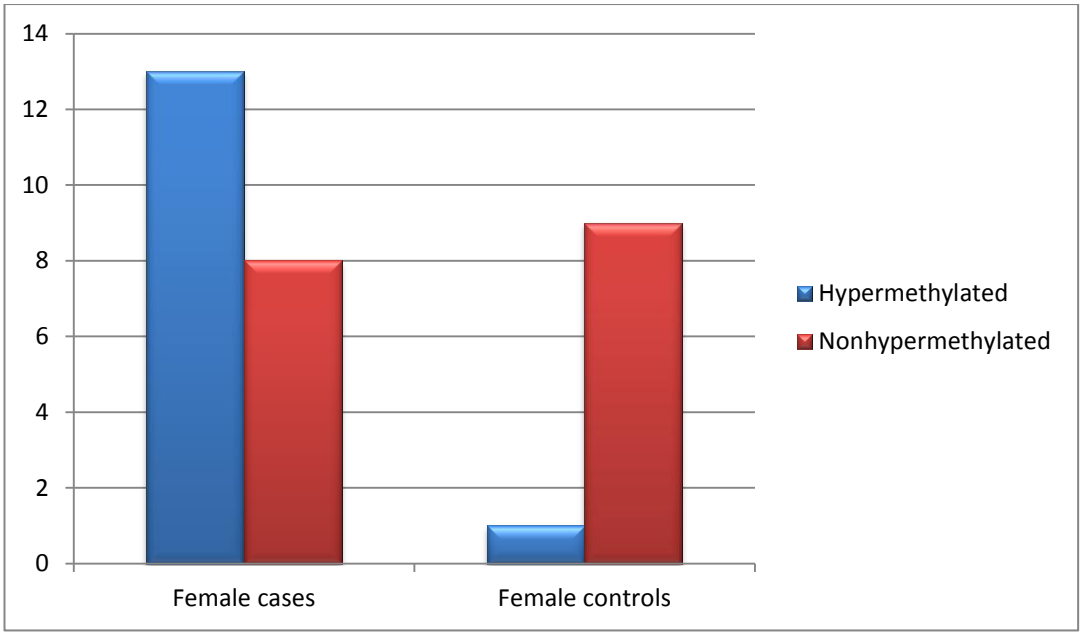

Fig.10: Histogram representing hypermethylated and nonhypermethylated female colorectal cancer cases and histopathologically confirmed normal female cases

Table 9: Representing no. of cases showing promoter hypermethylation and non-hypermethylation in male and female colorectal cancer patients during MSP amplification confirmed by 2% agarose gel electrophoresis

TOTAL CASES (50)

MALES (29)

PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATED	20	68.97% (20/29)
NON HYPERMETHYLATED	9	31.03% (9/29)

FEMALES (21)

PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATED	13	61.90 % (13/21)
NON HYPERMETHYLATED	8	38.09 % (8/21)

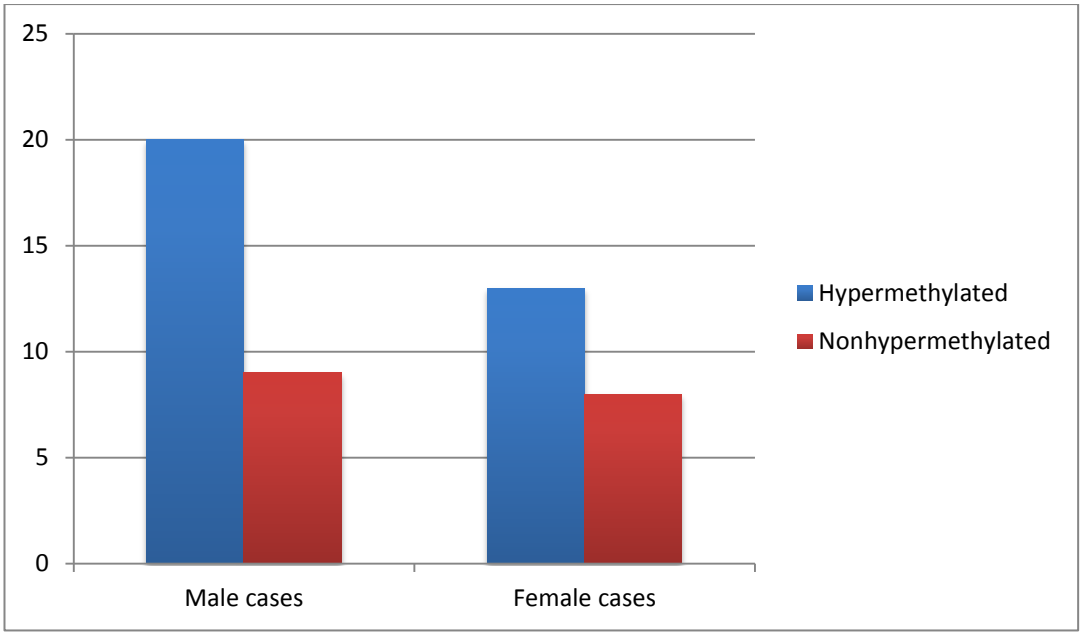

Fig.11: Histogram representing hypermethylated and nonhypermethylated male and female histopathologically confirmed colorectal cancer cases

4.3.2. Relationship of promoter hypermethylation of *p16* gene in Stage I/ II and Stage III/IV

There were 32 cases who were in Stage I and Stage II of the disease. Among these cases 18 cases were hypermethylated and 14 cases were nonhypermethylated. However, among 18 cases who were in Stage III and Stage IV of the disease, 15 cases were hypermethylated and 3 cases were nonhypermethylated (Table 10 and Fig.12). When the frequency of *p16* promoter hypermethylation was compared with clinical staging of the disease, *p16* promoter hypermethylation was found to be certainly higher in Stage III/IV (83.33%) compared to Stage I/ II (56.25%) (Table 10 and Fig.12) but the difference was not statistically significant (P =0.0673, Odds ratio=3.889 and 95% C.I=0.9370 to 16.14) (Fisher's exact test).

4.3.3. Relationship of promoter hypermethylation of *p16* gene and histological grade

The frequency of *p16* methylation was 54.54% (6/11) in well differentiated adenocarcinoma (WDAC), 67.74% (21/31) in moderately differentiated adenocarcinoma (MDAC) and 75% (6/8) in poorly differentiated adenocarcinoma (PDAC) (Table 11 and Fig.13). So, the degree of *p16* promoter hypermethylation increased with the increasing severity of the lesion but the difference was not again statistically significant (P =0.6145) (Chi square test).

Table 10: Representing no. of cases showing promoter hypermethylation and non-hypermethylation in stage I/II and stage III/IV during MSP amplification confirmed by 2% agarose gel electrophoresis

TOTAL CASES (50)

STAGE I/II (32)

PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATED	18	56.25 % (18/32)
NON HYPERMETHYLATED	14	43.75% (14/32)

STAGE III/IV (18)

PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATED	15	83.33 % (15/18)
NON HYPERMETHYLATED	3	16.66% (3/18)

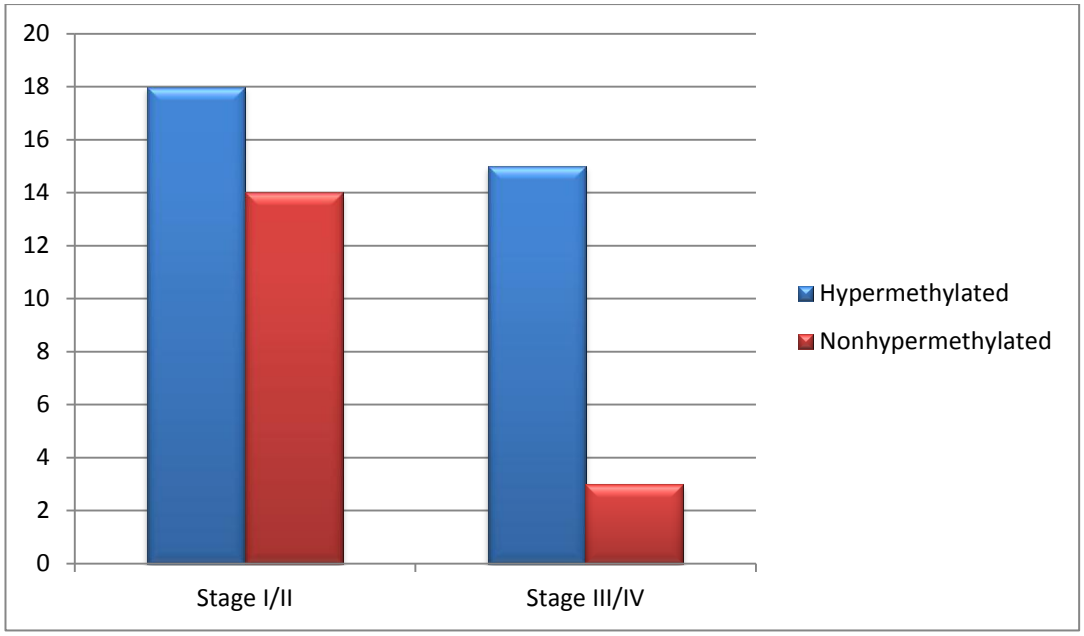

Fig.12: Histogram representing hypermethylated and nonhypermethylated cases of Stage I/II and Stage III/IV

Table 11: Representing no. of cases showing promoter hypermethylation and non-hypermethylation in well differentiated, moderately differentiated and poorly differentiated cases during MSP amplification confirmed by 2% agarose gel electrophoresis

TOTAL CASES (50)

WELL DIFFERENTIATED(11)

PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATED	6	54.54 % (6/11)
NON HYPERMETHYLATED	5	45.45% (5/11)

MODERATLY DIFFERENTIATED(31)

PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATED	21	67.74 % (21/31)
NON HYPERMETHYLATED	10	32.25 % (10/31)

POORLY DIFFERENTIATED(8)

PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATED	6	75 % (6/8)
NON HYPERMETHYLATED	2	25% (2/8)

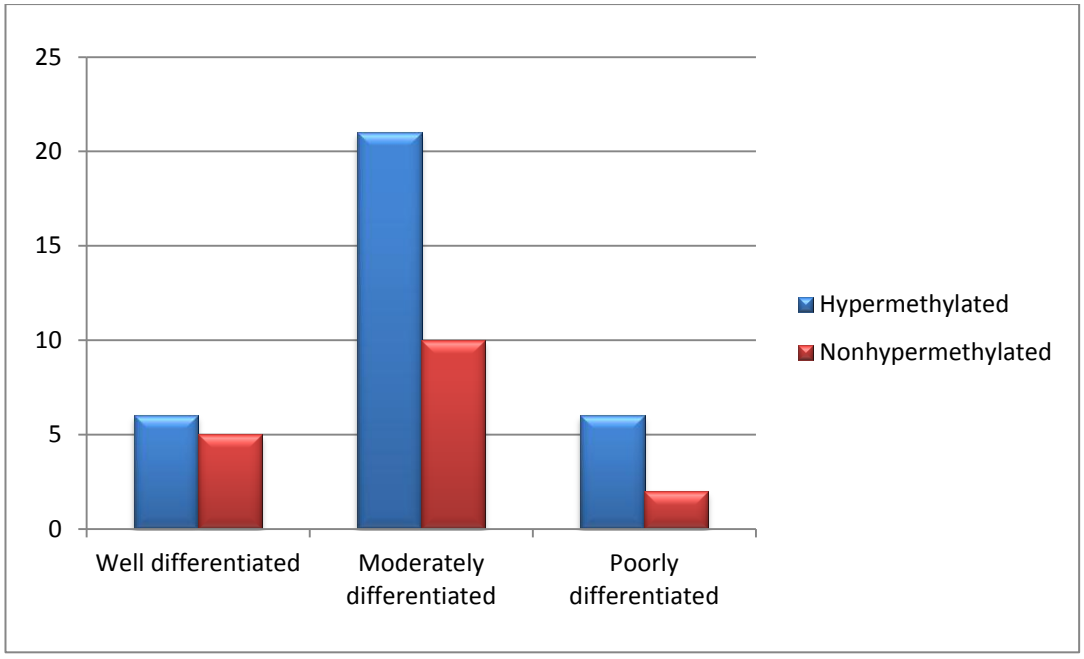

Fig.13: Histogram representing hypermethylated and nonhypermethylated cases of well differentiated, moderately differentiated and poorly differentiated adenocarcinoma

Discussion :

Colorectal cancer (CRC), commonly known as bowel cancer is the third most common cause of cancer-related deaths in the western world. Colorectal cancer is one of the most aggressive malignancies and occurs at a high incidence in most countries (Greenlee *et al*., 2000). The annual incidence of CRC worldwide has been estimated to be at least half a million (Kemp *et al*., 2004). It is a commonly diagnosed cancer in both men and women. In 2008, an estimated 148,810 new cases were diagnosed, and 49,960 deaths from colorectal cancer occurred (ACS, 2008). Most colorectal cancers (CRC) develop through multiple mutations in the normal colonic mucosa, and evolve through the adenoma-carcinoma sequence (Fearon *et al*., 1990 and Kinzler *et al*., 1996).The development of CRC is a multi-step process, which can arise due to cumulative effect of mutations in various different oncogenes, tumor suppressor genes and/or from epigenetic changes in DNA (Mustafa *et al*., 2007). Recent progresses made in the field of molecular biology have shed light on the different alternative pathways involved in the colorectal carcinogenesis, and more importantly cross talk among these pathways (Risques *et al*., 2003 and Takayama *et al*., 2006). Various endogenous and exogenous agents from environmental exposures are constantly damaging DNA, and in combination with low DNA repair capacity this have been interpreted as increasing the likelihood of cancer development (Wei *et al*., 1995;1996; Cheng *et al*., 1998; Shi *et al*., 2004).

Colon being the waste processor of our body is in particular exposed to a wide array of endo as well as exogenous chemicals. Normally detoxification reactions are going on in our body at all the times and sometimes these lead to the production of certain harmful chemical compounds e.g, Lithocholic acid (LCA) (component of bile acids) is implicated in human and experimental animal carcinogenesis. Its effect on apoptosis and proliferation of the colonic epithelium was studied in a 1, 2-dimethylhydrazine (DMH)-induced murine carcinogenesis model (Vassilikki *et al*., 2000). On the other hand we eat a variety of compounds both natural and manmade and these contain a cocktail of chemicals which have a potential of acting as carcinogens like mono sodium glutamate, poly aromatic hydrocarbons, nitrosamines (Siddiqi *et al*., 1992). As colon is always in direct contact with these types of chemicals it can be

understood as to why there is a high occurrence of colorectal cancer in general population.

One of the most important treatment of this fatal cancer is surgery and subsequent chemotherapy and radiotherapy. For this purpose, it is important to identify the occurrence of genetic alterations as a new parameter to estimate the malignancy of the cancer.

Tumor suppressor genes were initially hypothesized to be inactivated in cancer cells as a result of genetic defects of both alleles (i.e., the Knudson two-hit hypothesis). However, there is now evidence that epigenetic events, such as hypermethylation of cytosine–guanine (CpG) sites in regulatory regions (e.g., the promoter), may be a critical alternative mechanism of tumor suppressor gene inactivation. Epigenetic mechanisms of gene inactivation, including promoter hypermethylation, are undoubtedly important in cancer development and represent an alternative means of inactivating tumor suppressor genes. Nevertheless, the standard of proof for establishing that hypermethylation of the promoter of any given gene has a critical role in loss of gene expression and cancer development should probably be set quite high, regardless of whether the gene is a well-established tumor suppressor gene, like *p16*, or a potential tumor suppressor gene. However, before accepting the conclusion that hypermethylation of tumor suppressor gene promoters is invariably the cause of gene inactivation, it is worth evaluating the data a bit more critically.

The current study was aimed:

- To study the promoter hypermethylation status of *p16* gene in colorectal cancer subjects of Kashmiri origin and correlate them with histopathologically confirmed controls.

DNA methylation involves addition of a methyl group to the carbon 5 position of the cytosine ring. This reaction is catalyzed by DNA methyltransferases in the context of the sequence 5'-CG-3', which is also referred to as a CpG dinucleotide (Singal and Ginder, 1999; Jones and Laired, 1999). The methylation of gene, particularly the methylation of CpG-rich promoters, could block transcriptional activation (Singal and Ginder, 1999; Jones and Laired,

1999). A family of three enzymes, the cytosine-DNA methyltransferases, catalyzes methylation of the 5th position of the cytosine ring, using S-adenosylmethionine as the donor molecule. When this occurs within a CpG island located in the promoter region of a gene, it is also accompanied by changes in chromatin composition around the island that denies access to regulatory proteins needed for transcription. The chromatin structure is modified during gene silencing by affecting acetylation, phosphorylation, methylation, and/or ubiquitylation of histone tails (Jones and Baylin, 2002 and Kelly *et al*., 2002). Transcriptional silencing by CpG island hypermethylation affects genes involved in all aspects of normal cell function and now rivals genetic changes that affect coding sequence as a critical trigger for neoplastic development and progression (Jones and Laird, 1999; Baylin and Herman, 2000). The rapid advance in the study of gene-promoter hypermethylation in cancer was facilitated by the development of the methylation specific PCR (MSP) assay that allows for rapid detection of methylation in genes through the selective amplification of methylated alleles within a specific gene promoter (Herman *et al*., 1996). Gene promoter hypermethylation has become a target for developing strategies to provide molecular screening for early detection, diagnosis, prevention, treatment, and prognosis of cancer. The effectiveness of gene promoter hypermethylation for cancer screening and diagnosis ideally requires genes whose dysfunction occurs early in tumour development, are specific to a particular cancer, and a biological fluid or access to tissue that is specific to the disease being assessed. For the majority of cancers, it is difficult to meet all three of these criteria. This approach involves the detection of gene promoter regions that are aberrantly hypermethylated in human tumours. This change is associated with an epigenetically mediated gene silencing that constitutes an alternative to coding region mutations for loss of gene function (Jones and Baylin, 2002; Herman and Baylin, 2003). Gene silencing involves the modification of both the genetic and histone code (Jones and Baylin, 2002).

P16 tumour suppressor gene plays a monitor role in the passage of cells through the G1 to S phase of the cell cycle by binding to cyclin-dependent kinase 4 and inhibiting its effect on cyclin D1 (Merlo *et al*., 1995; wiser *et al*., 1999; Yu and Huang, 1999). The exon 1 coding sequences of the *p16* gene

resides within 5' CpG islands. This area is not methylated in most normal tissues but methylated in many human cancers. Methylation of cytosine residues at CpG sites in *p16* gene promotor, resulting in a silenced *p16* expression, has been reported in many cell lines, including CRC, and some primary carcinomas in varied origins, such as colon, brain, breast, bladder, ovary, lung, and myeloma and so on (Esteller *et al*., 1999). The *p16* methylation is common in CRC cell lines (Guan *et al*., 1999) and has been suggested to involve also in the primary CRC (Guan *et al*., 1999). Although most of authors hold the traditional viewpoint that detectable *p16* methylation necessarily link to the inactivation of *p16* protein, or transcriptional silencing of *p16* gene (Baylin *et al*., 1998), coexistence of *p16* methylation and *p16* expression in one specimen has been frequently described (Wiencke *et al*., 1999). The elucidation of the relationship between *p16* gene methylation and CRC will certainly help better understand the role of methylation of tumour suppressor genes in carcinogenesis. In the present study, frequent methylation of *p16* was observed in colorectal cancer. In this study, *p16* methylation was significantly associated with CRC. This result suggested that *p16* methylation might link to a more malignant outcome of CRC.

In the present study MSP was used for analysis of the methylation status of *p16* gene. This method provided significant advantages over previous ones used for assaying methylation. MSP is much more sensitive than Southern analysis, facilitating the detection of low numbers of methylated alleles and the study of DNA from small samples. MSP allows examination of all CpG sites, not just those within sequences recognized by methylation sensitive restriction enzymes. Fresh human tumour samples often contain normal and tumour tissue, making the detection of changes specific for the tumour difficult. However, the sensitivity of MSP suggests it would be useful for primary tumours as well, allowing for detection of aberrantly methylated alleles even if they contribute relatively little to the overall DNA in a sample.

Considering the important role of promoter methylation in inactivation of *p16* which is one of the most frequently altered genes in squamous cell carcinoma of oesophagus and many other human cancers, in the present study, the level of

p16 promoter methylation was investigated in colorectal carcinoma tissues of patients from Kashmir valley where frequency of colorectal cancer is higher.

There were 50 histopathologically confirmed cancer cases and 20 histopathologically confirmed normal cases as controls. Out of 50 cases 29 were males which corresponded to 58% and hence 21 cases out of 50 were females which corresponded to 42%. The male to female ratio of the cancer came to 1.38. However, among normal cases 10 were males and 10 were females with male to female ratio of 1. All the patients were symptomatic at the time of diagnosis. Clinicopathological data revealed that the patients presented with abdominal pain, change in bowel habits, rectal bleeding and loss of appetite. The other signs and symptoms were subjective weight loss, abdominal mass, vomiting or abdominal distention and anemia. Of all the ten districts highest number of colorectal cases turned out from Srinagar and Budgam districts of Kashmir.

The distribution of tumor was 60% (n = 30) in colon and 40% (n = 20) in rectum. Among colon cases 26% (n = 13) in sigmoid colon, 16 %(n=8) in ascending colon, 8 %(n=4) in transverse colon and 10% (n = 5) in descending colon. According to the classification of International Union against Cancer, there were 7 patients (14%) in stage I, 25(50%) in stage II, 11 (22%) in stage III and 7 (14%) in stage IV disease. Metastasis was found in 7 patients with distant metastasis in liver (n = 4) and lymph nodes (n = 5). All tumours were adenocarcinomas and on histological examination, 11 were well differentiated, 31 moderately differentiated and 8 poorly differentiated adenocarcinomas.

The genetic analysis of the cases and controls revealed that unlike other high risk regions, Kashmiri population has a different hypermethylation profile of *p16* promoter. It was found that two-third i.e. 66% (33/50) of the cases had *p16* promoter hypermethylation while as one-third i.e. 33% (17/50) of the cases were nonmethylated. The study also reveals that 20% (4/20) of the controls also had promoter hypermethylation of CpG islands of *p16* gene and 80% (16/20) did not showed promoter hypermethylation of CpG islands of *p16* gene. It was noticeable that, in this study, band U, i.e. PCR products amplified by primer U, was visible not only in 17 CRC cases and 16 controls which

showed unhypermethylated *p16* but also in few cases among other CRC specimen. Similar results were also reported by other investigators (Wiencke *et al*., 1999). This might first be attributed to the contamination of non-neoplastic cells that naturally harbored unmethylated DNA that could be derived from normal adjoining mucosal cells and from normal constituents in the stroma such as vascular endothelial cells, smooth muscles, fibroblasts and inflammatory cells. However, Guan *et al.*, 1999, demonstrated that band U appeared even in the samples acquired by micro dissection which got rid of the non-neoplastic cells (Guan *et al*., 1999). This indicated the possibility that tumour cells in one sample were virtually the mixture of those contained methylated and unmethylated DNA. The association of promoter hypermethylation with colorectal cancer was found to be significant.

The relationship between the promoter hypermethylation of *p16* gene and several clinicopathological parameters was examined. These parameters included gender, clinical staging and histological grade. Occurrence of *p16* methylation was found to be unequally distributed in males and females with more frequency in males than in females. Among 29 males, 20 cases were hypermethylated and 9 cases were unhypermethylated. However among 10 male controls 2 cases were hypermethylated and 8 cases were nonhypermethylated. The association of promoter hypermethylation with colorectal cancer was found to be significant in males. In comparison, among 21 females, 13 cases were hypermethylated and 8 cases were nonhypermethylated. However among 10 female controls 1 case was hypermethylated and 9 cases were nonhypermethylated. The association of promoter hypermethylation with colorectal cancer was again found to be significant in females too. Also on comparing the male cases with female cases, 20 cases were hypermethylated and 9 cases were unhypermethylated in males and 13 cases were hypermethylated and 8 cases were unhypermethylated in females, occurrence of *p16* methylation was found to be unequally distributed in males and females with more frequency in males than in females but the difference was not statistically significant.

Among the 32 patients who were in stage I and stage II, 18 cases showed promoter hypermethylation and 14 cases were nonhypermethylated. However among 18 patients who were in stage III and stage IV, 15 cases showed promoter hypermethylation and 3 cases were nonhypermethylated. When the frequency of *p16* promoter hypermethylation was compared with clinical staging of the disease, *p16* promoter hypermethylation was found to be certainly higher in Stage III/IV (83.33%) compared to Stage I/ II (56.25%) but the difference was not statistically significant.

Status of *p16* promoter hypermethylation was not found to correlate well with histological grades of the disease. Among 11 cases who were histologically well differentiated adenocarcinoma cases, 6 cases i.e., 54.54% (6/11) showed promoter hypermethylation and 5 cases were nonhypermethylated. From 31 moderately differentiated adenocarcinoma cases, 21 cases i.e., 67.74% (21/31) showed promoter hypermethylation and 10 cases were nonhypermethylated and among 8 poorly differentiated adenocarcinoma cases 6 cases i.e., 75% (6/8) showed promoter hypermethylation and 2 cases were nonhypermethylated. So, the degree of *p16* promoter hypermethylation increased with the increasing severity of the lesion but the difference was not statistically significant.

The study on colorectal cancer showed that more than 50% tissues expressed methylated *p16* promoter. Therefore, it is quite possible that like other geographical regions, methylation of promoter of *p16* gene is the major epigenetic event in colorectal cancer in the Kashmir valley. We observed completely methylated *p16* promoter in 33 cases out of 50. Though there was no selection bias in sampling, occurrence of *p16* methylation was found to be unequally distributed in males and females with more frequency in males than in females. Our study also showed that *p16* promoter hyper methylation was found to be certainly higher in Stage III/IV compared to Stage I/ II but the difference was not statistically significant. The study also showed that the degree of *p16* promoter hypermethylation increased with the increasing severity of the lesion but the difference was not again statistically significant. This study from India demonstrates that *p16* promoter methylation is a frequent

epigenetic event in colorectal cancer of the Kashmir region. These results also indicate that *p16* aberrant methylation may play an important role in colorectal cancer development. Therapeutic strategies targeting promoter hypermethylation may be highly beneficial in the Kashmiri population and other specific regions where incidence of colorectal cancer is associated with high frequency of *p16* promoter methylation. The data gives a clue that *p16* gene expression can be readily and fully restored and growth rate of cancer cells decreased by treatment of cancer cells with demethylating agents and DNA methylation inhibitors. The administration of drugs such as cytosine analogs might be able to restore the function of these tumour suppressor genes and slow the rate of colorectal cancer progression. It also demonstrates that hypermethylation of *p16* gene can be designated as epigenetic biomarker for the screening, diagnosis and prognosis of colorectal 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™ 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:

Agrose 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	20.0g

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.

Ehedium 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

Deoxyribosenucleotidetriphosphate (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 $MgCl_2$)

Primers: 100pM in sterile deionised water (Genescript)

100bp DNA ladder (0.5µg/µl)