Elucidation of Etiology of Colorectal Cancer: A Study on Silencing of MGMT Gene by Promoter Hypermethylation

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

By

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2.1 Colorectal Cancer

Colorectal cancer also known as bowel cancer is characterized by the uncontrolled growth of abnormal cells inside the colon and or rectum. The colon and rectum are parts of the digestive system also called the gastrointestinal or GI system. The colon is the site where water and salt from solid wastes are extracted before they are eliminated from the body. It is the longest part of large intestine and is approximately 1-1.5 m long. It is divided into 4 sections. The first section called the ascending colon begins where the small intestine attaches to the colon and extends upward on the right side of a person's abdomen. The second section is called the transverse colon as it crosses the body from the right to the left side. The third section, the descending colon, continues downward on the left side. The fourth section which is S shaped is known as the sigmoid colon. The sigmoid colon joins the rectum, which is the temporary storage place for the feces and is approximately 12-15 cm in length. The rectum in turn joins the anus. Histologically the colon and rectum wall comprises four distinct layers: mucosa, sub mucosa, muscle (muscularis propria) and serosa.

The most common type of colorectal cancer cell is adenocarcinoma which accounts for 95% of cases. Other, rarer types include lymphoma and squamous cell carcinoma. Adenocarcinoma is a malignant epithelial tumor, originating from glandular epithelium of the colorectal mucosa. Colorectal cancer usually develops slowly over a period of many years. Before a true cancer develops, it usually begins as a noncancerous polyp, which may eventually change into colorectal cancer. A polyp is a growth of tissue that develops on the lining of the colon or rectum. Certain kinds of polyps, called adenomatous polyps or adenomas (benign neoplasm) are most likely to become cancers, although majority of adenomas never develop into cancer. Once cancer forms in the large intestine, in time it can grow through the lining and into the wall of the colon or rectum. Cancers that have invaded the wall can also penetrate blood vessels or lymph vessels. Cancer cells typically spread first into nearby lymph nodes and can also be carried in blood vessels to the liver or lungs, or can spread in the abdominal cavity and to other areas. The extent to which a colorectal cancer has spread is described as its stage. Tumors that have not yet begun to invade the wall of the colon or rectum are called carcinomas *in situ*, and are not counted in cancer statistics.

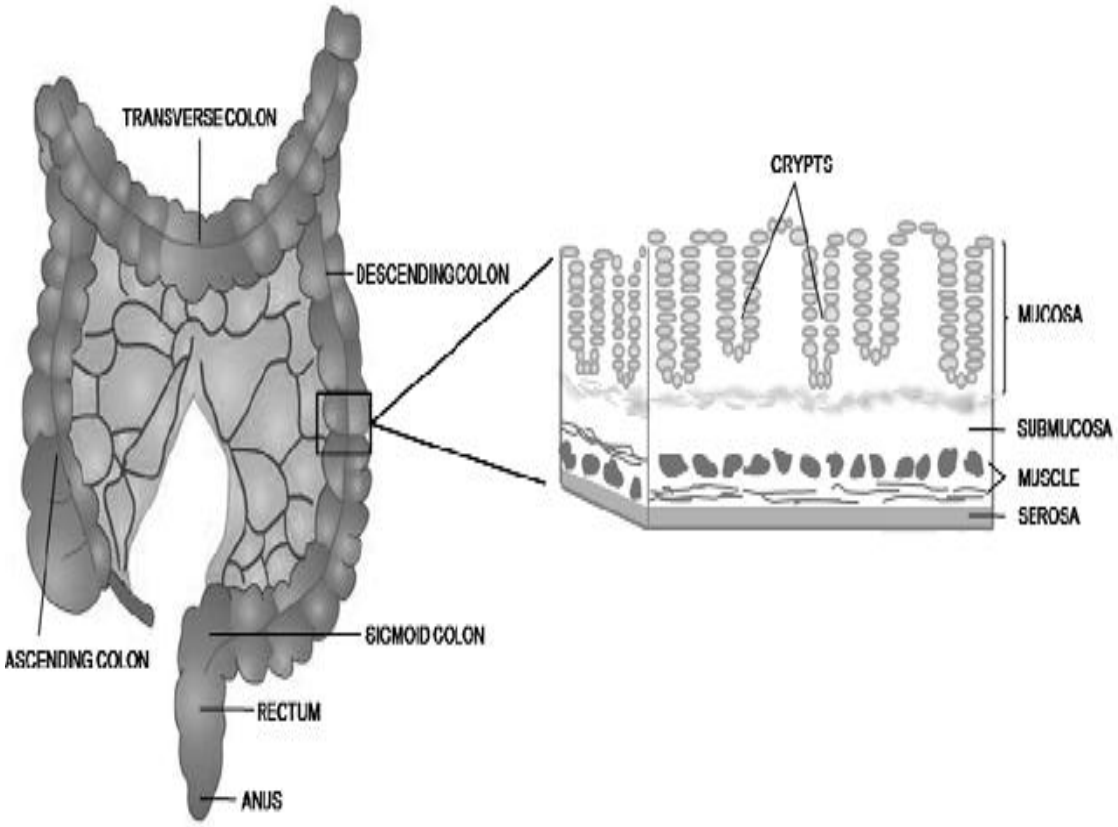

Figure 1: Normal colon and rectum.

Table1: TNM staging for CRC

AJCC Stage	**TNM stage**	**TNM stage criteria for colorectal cancer**
Stage 0	Tis N0 M0	Tis: Tumour confined to mucosa; cancer-*in-situ*
Stage I	T1 N0 M0	T1: Tumour invades submucosa
Stage I	T2 N0 M0	T2: Tumour invades muscularis propria
Stage II-A	T3 N0 M0	T3: Tumour invades subserosa or beyond (without other organs involved)
Stage II-B	T4 N0 M0	T4: Tumour 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.

There is good morphological and molecular genetic evidence that colon carcinoma develops through several precursor stages. The histological progression of colorectal cancer from adenoma to carcinoma is now recognized as the process through which most colorectal carcinomas develop and was first described by Morson and colleagues (Muto *et al.*, 1975) and the genetic pathway which parallels this transition has been suggested by Fearon, Vogelstein, Bodmer and others (Fearon and Vogelstein, 1990). Histological evidence of dysplasia is usually taken as the first recognizable step in the adenoma-carcinoma sequence. Small areas of epithelium with irregular glandular architecture termed aberrant crypt foci (ACF) have been reported to harbor mutations in *APC*, K-*ras* and *TP53* (Jen *et al.* 1994). However, the relationship between aberrant crypt foci and adenomas is still not clear. Progression of an adenoma is more likely with increased size, severe features of dysplasia villous rather than tubular architecture. Finally overt features of carcinoma are characterized by local invasion and eventual metastasis.

2.2 Epidemiology of colorectal cancer

Colorectal cancer (CRC) is one of the most common cancers in developed countries. Colorectal cancer is the third most commonly reported cancer in the world, with over one million cases occurring annually (Parkin *et al.*, 2005; Garcia *et al.*, 2007). More than 600,000 people die every year from the disease worldwide (Garcia *et al.*, 2007). It is the second most common cause of death from cancer across all cancer types in men and women in Europe (Ferlay *et al.*, 2007). In 2008, colorectal cancer ranked 3rd among the most incident forms of non-skin cancer in the U.S. for both men and women (Jemal *et al*., 2008). Approximately 146,970 new cases of large bowel cancer are diagnosed each year in US, of which 106,100 are colon and the remainder rectal cancers (Jemal *et al.*, 2009).Annually, approximately 49,920 Americans die of CRC, accounting for approximately 9% of all cancer deaths. Over one-third (329,529 cases -36%) of new cases of colorectal cancer occur outside industrialized countries, the standard myth of colorectal cancer being a disease restricted to western countries needs to be dispelled. The incidence of this malignancy shows considerable variation among racially or ethnically defined populations in multi racial/ethnic countries. It is of potentially considerable significance that the high rates are to be found in a variety of population groups including Blacks in Detroit (34.9 per 100,000), Los Angeles (34.8), San Francisco (33.8), Atlanta (32.4) and New Orleans (31.4), Japanese and Whites in Hawaii (34.4 and 32.7,respectively) and non-Maori in New Zealand (31.2). Incidence rates (per 100,000) reported for Blacks, Whites, Asian/Pacific Islanders,

American Indian/Alaskan and Hispanics are 50.1, 42.9, 38.2, 28.6 and 28.4 respectively (Howe *et al.*, 2001). World-wide, in men the lowest incidence rates are found in a variety of population groups in the non-industrialized countries with the lowest rate reported in Setif, Algeria (0.4 per 100,000). In women, the group of highest incidence rates includes population groups in New Zealand and North America with the lowest rates recorded in Algeria and India. In each sex, a number of low rate regions are found in India (Parkin *et al.*, 1997).

2.3 Risk factors for colorectal cancer

Epidemiologic studies have revealed a number of risk factors for colorectal cancer including age, family history of colon cancer or inflammatory bowel disease, smoking, alcohol consumption, obesity, diet etc.

2.3.1 Age

Colorectal cancer is most commonly found in those aged 50 years and over. The SEER, 2009 (Surveillance Epidemiology and End Results) Cancer Statistics Review found that between 1998 and 2002, less than 15% of new colorectal cancer cases occurred among those less than 54 years of age, 17% occurred among those between 55 and 64 years old, 26.3% occurred among those between 65 and 74 years old, 29.2% occurred among those between 75 and 84 years old, and 12.6% of new cases were among those over the age of 85 (SEER, 2009). When colon and rectal cancers are considered separately, over 50% of new cases in each group still occur among those 65 to 84 years old (SEER, 2009).

2.3.2 Sex

Men are more likely than women to develop colorectal cancer. The incidence rate of colorectal cancer between 2000 and 2004 was 69.2 per 100,000 population among men and 45.8 per 100,000 populations among women (Jemal *et al.*, 2008). The SEER, 2009 Review found similar incidence rates between 1998 and 2002. Additionally, the breakdown between the rates of colon and rectal cancers were given separately in that report. The incidence of colon cancer was 43.4 per 100,000 among men and 34.5 per 100,000 among women and the

incidence of rectal cancer was 18.5 per 100,000 and 11.7 per 100,000 for men and women respectively.

2.3.3 Race

African Americans have the highest incidence rates of colorectal cancer. Between year 2000 and 2004 the number of new cases of colorectal cancer per 100,000 population among men was 60.4 among whites, 72.6 among African Americans, 49.7 among Asian Americans and Pacific Islanders, 42.1 among American Indians and Alaska Natives and 47.5 among Hispanics and Latinos (Jemal *et al.,* 2008). Among women during the same time period, the numbers of new cases of colorectal cancer per 100,000 populations were 44.0 among whites, 55.0 among African Americans, 35.3 among Asian Americans and Pacific Islanders, 39.6 among American Indians and Alaska Natives, and 32.9 among Hispanics and Latinos (Jemal *et al.*, 2008).

2.3.4 Physical Activity

Studies have also found that physical activity is associated with lower risk of colorectal cancer. A meta-analysis published in 2004 found that among 47 studies that examined the association between colon and/or colorectal cancer and physical activity, physically active males had a lower risk of developing colon cancer regardless of specific type of activity (recreational or occupational) and regardless of the type of study (cohort or case-control) (Samad *et al.*, 2005). The study found that women who participated in recreational physical activity also had a lower risk of developing colon cancer in both cohort and case-control studies but that occupational physical activity among women only showed a reduced risk for colon cancer among case-control studies. Additionally, this meta-analysis found no association between physical activity and risk of rectal cancer. Another study examined physical activity based on data from the European Prospective Investigation into Cancer and Nutrition (EPIC) (Friedenreich *et al.*, 2006). A test for heterogeneity found no statistically significant difference in the results for men and women for overall physical activity and each type of physical activity (occupational, household, and recreational) in their association with colon and rectal cancers. Further analysis showed that physically active adults were at a reduced risk for colon cancer compared to inactive adults (RR = 0.78; 95% CI 0.59 – 1.03) with recreational activity being the most protective form of physical activity. This study also found that increased physical activity was not associated with a reduction in rectal cancer risk. While this study showed no difference in the association between physical activity and

colon cancer risk among men and women, other studies have found that physical activity is not associated or only modestly associated with reduced risk of colon cancer in women (Calton *et al.*, 2006 ; Mai *et al.*, 2007). The conflicting results of these studies are evidence that further research is needed to determine the true associations if any between physical activity and colon cancer risk. One aspect of physical activity that has not been considered in the majority of the studies is looking at physical activity and colorectal cancer risk is lifetime change in physical activity. A case-control study conducted at the German Cancer Research Center collected data about recreational and occupational physical activity at ages 20, 30, 40, 50, and 60 years for each subject. The results of the study showed that the odds ratio was 0.26 (95% CI 0.08 – 0.84) for colon cancer risk for lifelong constantly high-exercisers compared with lifelong non-exercisers (Steindorf *et al.*, 2005). This study found no association between lifetime physical activity and risk of rectal cancer. Further analysis of lifetime physical activity patterns including type, frequency and duration will strengthen our understanding of the potential association between physical activity and colon cancer risk.

2.3.5 Obesity

Over the past 30 years the rates of obesity have increased dramatically. The prevalence actually doubled among adults aged 20 years and older between the years 1980 and 2002 (Hedley *et al.*, 2004). A recent study of the prevalence of obesity in the United States analyzed data from the National Health and Nutrition Examination Survey (NHANES) from 2003 to 2004. The researchers used body mass index (BMI) the ratio of weight in kilograms to the square of height in meters in order to calculate rates of overweight and obesity. The World Health Organization has set forth a standard definition of each weight class based on BMI. The results of the study showed that based on these definitions in 2003-2004, 61.8% of adult women were considered overweight or obese. The prevalence of obesity (BMI of greater than 30 kg/m2) was 33.2% and extreme obesity (BMI of greater than 40 kg/m2) was 6.9%. Non-Hispanic black women had the highest prevalence for all categories: overweight or obese, obese, and extremely obese at 81.6%, 53.9%, and 14.7% respectively (Hedley *et al.*, 2004). These rates are alarming considering the increased risk of morbidity and mortality associated with a higher BMI (Hedley *et al.*, 2004).

A number of studies have shown that being overweight is associated with increased risk of colorectal cancer. A case-control study conducted by Caan found that men who had a BMI in the highest quintile were almost 2 times as likely to develop colon cancer as men with a

BMI in the lowest quintile (OR = 1.96; 95% CI 1.50 – 2.57). Women with a BMI in the highest quintile were about 1.5 times as likely to develop colon cancer when compared to women with BMIs in the lowest quintile (OR = 1.45; 95% CI 1.08 – 1.94). The study also found that these associations were much stronger among younger men and women with a family history of colon cancer (Caan *et al.*, 1998). Another study looking the association between BMI and colon cancer risk found that those with a BMI ≥ 30.0 kg/m2 had an increased risk of developing colon cancer compared to those with a normal BMI (OR = 1.54; 95% CI 1.03 – 2.31). The study also found that those whose BMI increased by more than 10 kg/m^2 between their 30s and the time of recruitment into the study had a higher risk of developing colon cancer than those whose BMI did not change as much (Nock *et al.*, 2008). Obesity is an important risk factor to consider based on the recent trends in the U.S. If there is a strong association between BMI and colon cancer risk, then continually increasing obesity rates will only increase the incidence of colon cancer throughout the population.

2.3.6 Smoking

The large bowel has not historically been considered as a site where the risk of cancer is linked to cigarette smoking (IARC, 1986) although it has been suggested that it may be an independent risk factor which may be specifically associated with the early stages of colorectal epidemiology (Giovannucci *et al.*, 1994). A more recent review of all epidemiological evidence has indicated the strength and consistency of this finding (Giovannucci, 2001). Giovannucci concluded that 21 out of 22 studies found that long-term, heavy cigarette smokers have a 2–3-fold elevated risk of colorectal adenoma (Giovannucci, 2001). The risk of large adenomas, those who present a high risk of colorectal cancer within a relatively short time frame was elevated in smokers in all 12 studies which examined this association. The studies of smoking and colorectal cancer risk conducted earlier in the 1950s through 1970s did not show consistently any association and led review groups to consider that based on the available evidence that there was no association demonstrated (IARC, 1986). However 27 studies in various countries, including the large majority of those that have been conducted in the past two decades, show a consistent association between tobacco use (essentially cigarette smoking) and colorectal cancer. In the US 15 of 16 studies conducted after 1970 in middle-age men and elderly men and in the 1990s in women demonstrate such an association. Giovannucci considered that this temporal pattern is consistent with an induction period of three to four decades between exposure and the

development of clinical colorectal cancer (Giovannucci, 2001). Overall accumulating evidence much within the past decade strongly supports the addition of colorectal cancer to the list of tobacco associated malignancies. Such an association has biological plausibility since carcinogens from tobacco could reach the colorectal mucosa through either the alimentary tract or the circulatory system and then damage or alter expression of cancer-related genes. It appears likely that up to one in five colorectal cancers in the US may be associated with such exposure.

2.3.7 Dietary and nutritional practices

For many years, a diet rich in vegetables and fruit has been associated with a reduced risk of colorectal cancer in many, but not all, observational studies (Steinmetz and Potter, 1991). The association between fruit and vegetable consumption and the incidence of colon and rectal cancers has been studied in two cohorts: the Nurses' Health Study (88,764 women) and the Health Professionals' Follow-up Study (47,325 men) (Michels *et al.*, 2000). Assessment of the diet was completed during different calendar years in women and men, during which a total of 1,743,645 person-years of follow-up were accrued and 937 cases of colon cancer were identified. No association was found between colon cancer incidence and fruit and vegetable consumption. For women and men combined, a difference in fruit and vegetable consumption of one additional serving per day was associated with a co-variate-adjusted RR of greater magnitude but lacking statistical significance (1.02; 95% CI, 0.98–1.05) (Steinmetz and Potter, 1991). This lack of association between consumption of vegetables and fruits and colorectal cancer risk contradicts a widely accepted relationship between nutritional practices and chronic disease risk. Another association under scrutiny is that between fat intake and colorectal cancer risk. Hitherto, there appeared to be consistent evidence from epidemiological studies that intake of dietary fat and meat is positively related to colorectal cancer risk: this evidence is obtained from ecological studies, animal experiments, and case-control and cohort studies. Many of these studies have failed to demonstrate that the association observed with fat intake is independent of energy intake.

The report by Willett provided the best epidemiological evidence to date identifying increased meat consumption as a risk factor for colon cancer independently of its contribution to fat intake and total caloric intake (Willett *et al.*, 1990). A recently published meta-analysis of 13 prospective studies looking at meat consumption and colorectal cancer risk has reported an increased risk (12–17%) with a daily increase of 100 g of all meat or red meat. The risk was higher (49%) with a daily increase of 25 g of processed meat

(Sandhu *et al.*, 2001). Again in a second study published in parallel, high intake of carcinogenic compounds produced when meat is well-cooked at high temperatures has been associated with an increased risk of colorectal adenomas (Sinha *et al.*, 2001).

Among protective dietary factors, the original hypothesis of the effect of dietary fiber was based on a clinical/pathological observation and a hypothesized mechanism whereby increasing intake of dietary fiber increases fecal bulk and reduces transit time. The term 'fiber' encompasses many components each of which has specific physiological functions. The commonest classification is into the insoluble, non-degradable constituents (mainly present in cereal fiber) and into soluble, degradable constituents like pectin and plant gums which are mainly present in fruits and vegetables. Epidemiological studies have reported differences in the effect of these components. For example Tuyns and Kune found a protective effect for total dietary fiber intake in case-control studies (Tuyns *et al.*, 1987 and Kune *et al.*, 1987) and the same was found in one prospective study (Heilbrun *et al.*, 1986). However a large number of studies could find no such protective effect (Willett, 1990). The large majority of studies in humans found no protective effect of fiber from cereals but consistently found a protective effect of fiber from vegetable and perhaps fruit sources (Willett, 1990; Steinmetz and Potter, 1991) and dietary diversity has been shown to be an important element in this protection (Fernandez *et al.*, 1996). This could conceivably reflect an association with other components of fruits and vegetables, with 'fiber' intake acting merely as an indicator of consumption. Two randomized studies conducted in the US looking at dietary interventions and the risk of recurrent adenomatous polyps have revealed no protective effect on the recurrence rate of colorectal polyps. The dietary interventions in question were for the Polyp Prevention Trial (Schatzkin *et al.*, 2000) to have either intensive counseling to follow a low-fat, high fiber, fruit and vegetable diet or to be given a brochure on healthy eating and for the Wheat Bran Fiber Study to have a high wheat bran fiber cereal supplement (13.5 g of fiber in 2/3 cup cereal per day) or low wheat bran fiber cereal supplement (2 g of fiber in 2/3 cup cereal per day). The latter study reports that increasing dietary fiber will not reduce the risk of developing colorectal cancer, however, praising the benefits of high fiber diets for the prevention of other chronic conditions. Possible explanations for the observed negative results in both studies may be a short follow-up period precluding the detection of cancerous lesions that require of longer time before emerging. A potential pathway for this protective association has been investigated in a novel epidemiological study design (Freedman *et al.*, 1996). Cruciferous vegetable intake exhibited a significant inverse association with colorectal cancer risk (OR, 0.59; 95% CI,

0.34, 1.02). When tumors were characterized by p53 over expression (p53 positive) etiological heterogeneity was suggested for family history of colorectal cancer (OR, 0.39; 95% CI, 0.16, 0.93), intake of cruciferous vegetables (test for trend, P = 0.12) and beef consumption (test for trend, P = 0.08). Cruciferous vegetable consumption exhibited a significant association when p53 positive cases were compared with controls (OR, 0.37; 95% CI, 0.17, 0.82). When p53 negative cases were compared with controls a significant increase in risk was observed for family history of cancer (OR, 4.46; 95% CI, 2.36, 8.43) and beef consumption (OR, 3.17; 95% CI, 1.83, 11.28). The p53 (positive) dependent pathway was characterized by an inverse association with cruciferous vegetable intake and p53 independent tumors were characterized by family history and beef consumption (Freedman *et al.*, 1996).

2.3.8 Heredity and Medical History

People who have a first-degree relative (parent, sibling, or offspring) who has had colorectal cancer have about twice the risk of developing the disease compared to individuals with no family history (Johns and Houslton, 2001; Butterworth *et al*, .2006). The risk increases even further if the relative was diagnosed at a young age or if there is more than one affected relative (Johns and Houslston, 2001). About 20% of all colorectal cancer patients have a close relative who has been diagnosed with the disease (Lynch and de la Chapelle, 2003). About 5%-10% of patients with colorectal cancer have an inherited genetic alteration that causes the cancer (Lynch and de la Chapelle, 2003).One such disorder is familial adenomatous polyposis (FAP); which causes hundreds or thousands of polyps to develop in the colon from a very young age. If these polyps are left untreated, that person has a very high risk of those polyps developing into cancer. Another genetic disorder that causes colorectal cancer is hereditary non-polyposis colorectal cancer (HNPCC) which accounts for 5%-8% of colorectal cancers (Heavey *et al.*, 2004). Unlike FAP, HNPCC does not result in an excess number of polyps. According to Heavey, HNCPP "seems to accelerate the carcinogenic process through an increased mutation rate in microsatellite regions, which then affects other genes involved in cell cycling and proliferation" (Heavey *et al.*, 2004). Someone with HNPCC has an 80% chance of developing colorectal cancer throughout his or her life. While other genetic mutations have been associated with increased colon cancer risk, those two (FAP and HNPCC) are the most common. While other factors also contribute to the development of colon cancer, understanding family history is an important first step in assessing an individual's risk.

2.3.8.1 A personal history of CRC, polyps, or chronic inflammatory bowel disease

People who have had colorectal cancer are more likely to develop new cancers in other areas of the colon and rectum, even if the first cancer has been completely removed. The risk of a second cancer is much greater if the first cancer was diagnosed at age 60 or younger. People who have had one or more adenomatous polyps have an increased risk of colorectal cancer. This is especially true if the polyps were large or if there was more than one (Schatzkin *et al.*, 1994). People who have a chronic inflammatory bowel disease of significant duration and involving the entire bowel have an increased risk of developing colorectal cancer (Bernstein *et al.*, 2001) This includes conditions such as ulcerative colitis and Crohn disease in which the colon is inflamed over a long period of time.

2.3.8.2 Diabetes

Many studies have found an association between diabetes and increased risk of colorectal cancer in both men and women. (Giovannucci, 2007; Larsson *et al.*, 2005) Adult onset (Type 2) diabetes, the most common type of diabetes, and colorectal cancer share similar risk factors, including physical inactivity and obesity. However, a positive association between diabetes and colorectal cancer has been found in studies that accounted for physical activity, body mass index, and waist circumference (Larsson *et al.*, 2005).

2.4 Molecular biology of CRC

In 1990 Fearon and Vogelstein proposed a multistep model for colorectal carcinogenesis that has become widely cited (Fearon and Vogelstein, 1990). Their model is based on four principle points. First, colorectal tumors are clonal entities that arise as the result of the mutational activation of oncogenes as well as the inactivation of tumor suppressor genes. Second, at least four to five genes must be mutated to accomplish carcinogenesis. Third, the final accumulation of mutations does not have to happen in a specific order. There is an order in which these mutations tend to accumulate, but the total accumulation, not the sequential order, is the key event. Last, some tumor suppressor genes, when mutated, can produce the neoplastic phenotype even when in the heterozygous state-knock-out of both alleles is not always necessary.

The two major pathways in CRC carcinogenesis are the chromosomal instability (CIN) and the microsatellite instability (MSI) pathways (Takayama *et al.*, 2006). The discovery of the

molecular changes that occur in the chromosomal instability pathway has been the foundation behind the development of the adenoma- carcinoma sequence. Most familial and sporadic CRCs develop in pre-existing adenomatous polyps (Piepins and Sandler, 1994). The development of the precancerous adenomatous polyp and its subsequent malignant degeneration involve multiple allelic losses resulting in loss of heterozygosity (LOH). These allelic losses lead to dysfunctional tumor suppressor genes or aberrant activation of proto-oncogenes (Robbins and Itzkowitz, 2002). The MIS pathway involves mutations in DNA mismatch repair (MMR) repair genes. The products of these genes are responsible for the recognition of DNA replication errors. Areas within the genome that contain tandem repeats of DNA sequences, also known as microsatellites, are particularly susceptible to replication errors. The occurrence of these replication errors within the coding sequence of genes responsible for cellular growth and regulatory functions can lead to malignant transformation (Radtke *et al.*, 2006). Recently, other molecular pathways involved in colorectal tumorogenesis, such as the transforming growth factor (TFG)-β signaling pathway, have been identified. Additionally, various alterations in expression of genes involved in tumor angiogenesis, growth, and metastasis have been described.

2.4.1 Chromosomal instability pathway

2.4.1.1 APC/β-catenin

One mutation known to occur early in the adenoma-carcinoma Sequence affects the adenomatous Polyposis coli (APC) tumor suppressor gene located on chromosome 5q21 (Bodmer *et al.*,1987; Groden *et al.*,1991). Germ-line APC mutations give rise to familial adenomatous polyposis, an inherited cancer-predisposition syndrome in which more than 100 adenomatous polyps can develop; in carriers of the mutant gene, the risk of colorectal cancer by the age of 40 years is almost 100% (Kinzler andVogelstein, 2002 ; Lynch *et al.*, 2008; Goss and Groden, 2000). The product of APC gene is a multifunctional protein with several domains that is known to interact with a number of other proteins including beta-catenin, glycogen synthase kinase (GSK)3B, end binding protein(EB) 1,Bub Kinase and human homologue of Drosophila discs large tumor suppressor protein (hDLG) (Rubinfeld *et al.*, 1996; Kaplan *et al.*, 2001; Matsumine *et al.*, 1996). APC mutation or allelic losses of 5q are observed in 40-80% of CRC (Vogelstein *et al.*, 1988, Powell *et al.*, 1992; Miyoshi *et*

al., 1992; Miyaki *et al.*, 1994) and are found at similar frequency in adenomas (Vogelstein *et al.*, 1988; Powell *et al.*, 1992; Miyoshi *et al.*, 1992; Miyaki *et al.*, 1994; Jen *et al.*, 1994). Interestingly although APC mutations occur at similar frequency at all stages of colonic tumor progression, allelic loss or loss of heterozygosity (LOH) has been shown to increase in frequency from earlier adenomas through to invasive carcinoma (Miyaki *et al.*, 1994). Furthermore mutated APC has been demonstrated in adenomas as small as 0.5cm (Powell *et al.*, 1992), reinforcing the belief that such mutations are involved earlier in adenoma-carcinoma sequence. Finally about half of those tumors with wild type APC have been found to harbor beta catenin mutations (Morin *et al.*, 1997; Sparks *et al.*, 1998) suggesting that these mutations can substituted for APC mutations in colorectal carcinogenesis. However it is intriguing that β-catenin mutations occur significantly more frequently in small adenomas (12.5 per cent) than in large adenomas (2.4 per cent) or invasive cancers (1.4 per cent) (Samowitz *et al.*, 1999). This observation suggests that adenomas with β-catenin mutations may be less likely to progress to invasive cancer.

2.4.1.2 p53

The p53 gene is the gene most frequently altered in human cancers (Caron and Soussi, 1992). Located on the short arm of chromosome 17, p53 was initially implicated in colorectal cancer as a result of the frequent loss of 17p in allelic loss and cytogenetic studies (Baker *et al.*, 1989; Fearon *et al.*, 1987; Meling *et al.*, 1993; Muleris *et al.*, 1985). In brief p53 has been labeled the "guardian of the genome" because of its ability to block cell proliferationin the presence DNA damage to stimulate DNA repair and to promote apoptotic cell death if repair is insufficient (Lane, 1992).The inactivation of the p53 pathway by mutation of *TP53* is the second key genetic step in colorectal cancer. In most tumors, the two *TP53* alleles are inactivated, usually by a combination of a mis-sense mutation that inactivates the transcriptional activity of p53 and a 17p chromosomal deletion that eliminates the second *TP53* allele (Kinzler and Vogelstein, 2002; Grady and Markowitz, 2002; Fearon and Bommer, 2008; Baker *et al.*, 1990). Wild-type p53 mediates cell-cycle arrest and a cell-death checkpoint, which can be activated by multiple cellular stresses (Vazquez *et al.*, 2008). The inactivation of *TP53* often coincides with the transition of large adenomas into invasive carcinomas (Baker *et al.*, 1990). In many colorectal cancers with mismatch-repair defects, *TP53* remains wild-type though in these cancers the activity of the p53 pathway is probably attenuated by mutations in the inducer of apoptosis (Kinzler and Vogelstein, 2002, Fearon and Bommer, 2008).Many reports on frequency of p53 mutations

in colorectal tumors have been based on the immuno-histochemical evidence of over expression, some have employed direct DNA sequencing and others have investigated 17p allelic loss. Thus it can be said that alteration in p53 or 17p allelic loss has been reported in 4-26 % of adenomas (Vogelstein *et al.*, 1988; Scott *et al.*, 1993; Rashid *et al.*, 1999; Darmon *et al.*, 1994; Ohue *et al.*, 1994; Yamaguchi *et al.*, 1994; Kaklamanis *et al.*, 1993) in approximately 50% of invasive foci within adenomatous polyps (Ohue *et al.*, 1994; Yamaguchi *et al.*, 1994) and in 50-75% of adenocarcinomas (Vogelstein *et al.*, 1988; Hardingham *et al.*, 1998; Darmon *et al.*, 1994; Kaklamanis *et al.* 1993; Kaserer *et al.*, 2000; Boland *et al.*, 1995). This distribution has led to the belief that functional inactivation of p53 protein is associated with the transition from adenoma to carcinoma.

2.4.1.3 K-ras

A further genetic alteration believed to occur early in the adenoma-carcinoma sequence is an activating mutation of the oncogene K-*ras*. This oncogene, one of three of the ras gene family encodes a 21-kDa protein (ras p21) involved in signal transduction of regulatory pathways critical for normal proliferation and differentiation (Bos, 1989; Bourne *et al.*, 1990). It is a Guanosine 5'- Triphosphate(GTP) binding protein located at the cytoplasmic aspect of cell membrane with intrinsic GTPase activity that is regulated by several other proteins (King, 2000). All known carcinogenic mutations of K-ras oncogene affect codons in the GTP-binding domain, decrease its GTPase activity and result in a constitutively active ras protein (King, 2000).

Activating K-ras mutations occur in 35-42 per cent of colorectal carcinomas (Vogestein *et al.*, 1988) and are observed at a similar frequency in large adenomas (Vogestein *et al.*, 1988; Scott *et al.*, 1993; Rashid *et al.*, 1999). The relatively high frequency of ras mutated adenomas supports the hypothesis of this oncogene as an early participant in the adenoma-carcinoma sequence. However as K-ras mutation is less common in small adenomas (Vogelstein *et al.*, 1988; Scott *et al.*, 1993; Rashid *et al.*, 1999) it seems that while this alteration confers a growth advantage it is unlikely to be an initiating factor in colorectal tumorigenesis.

2.4.1.4 DCC

Located on chromosome 18, the primary role of the product of the deleted in colon cancer (DCC) gene is believed to be tumor suppression and possibly as an apoptosis substrate (Robbins and Itzkowitz, 2002). Approximately 70% of CRCs exhibit losses on chromosome

18 (Fearon and and Vogelstein, 1990). Several late steps in the adenocarcinoma sequence are linked to loss of DCC function. Recently the significance of DCC in colorectal carcinogenesis has been questioned. Other genetic loci involved in CRC development, such as SMAD2 and 4 have been identified near the DCC sequence on chromosome 18q (Hahn *et al.*, 1996; Eppert *et al.*, 1996) . Loss of DCC may represent only a marker for deletion of adjacent tumor suppressor genes. Therefore the significance of DCC in colorectal tumorogenesis remains an area of debate and investigation.

2.4.1.5BRAF

Oncogenic mutations of BRAF, which activate the mitogen-activated protein kinase (MAPK) signaling pathway,occur in 13% of colorectal cancers (Davies *et al.*, 2002). *BRAF* mutations signal BRAF serine–threonine kinase activity which further drives the MAPK signaling cascade (Rajagopalan *et al.*, 2002; Siena *et al.*, 2009). *BRAF* mutations are detectable even in small polyps, (Nosho *et al.*, 2008) and as compared with *RAS* mutations; they are more common in hyperplastic polyps, serrated adenomas, and proximal colon cancers, particularly in those with the CIMP phenotype.

2.4.1.6 PI3K

One third of colorectal cancers bear activating somatic mutations in *PI3KCA*, which encodes the catalytic subunit of phosphatidylinositol 3-kinase (PI3K) (Samuels *et al.*, 2004). Less common genetic alterations that may substitute forPI3KCAmutations include loss of PTEN, an inhibitor of PI3K signaling, as well as amplification of insulin receptor substrate 2(IRS2), an upstream activator of PI3K signaling, and co-amplification of AKT and PAK4 which are downstream mediators of PI3K signaling (Parsons *et al.*, 2005).

2.4.2 Microsatellite instability Pathway

Recently an alternative pathway of tumorigenesis for a subset of colorectal tumors has been proposed, characterized by the presence of MSI. Microsatellites are a type of DNA that consists of tandem repeats usually between one and five base pairs, repeated many times (Wheeler and Bodmer, 2000). Hundreds of thousands of microsatellites are found interspersed throughout the human genome and are particularly prone to errors during DNA replication. Such errors are usually repaired by mismatch repair (MMR) proteins but in the absence of competent MMR function microsatellite errors accumulate (Wheeler and Bodmer, 2000).When these errors are sufficiently frequent the terms MSI or replication

error positive (RER^+) is applied. More recently tumours showing MSI have been further classified into those exhibiting low and high levels of instability (MSI-L and MSI-H respectively) (Dietmaier *et al.*, 1997). When a cell is MMR deficient it is not only microsatellites that are at risk of replication error but all nucleotide repeat sequences, including those in coding regions of key regulatory genes. Thus MSI can be interpreted as a marker for a state of hyper mutability or a 'mutator phenotype (Parsons *et al.*, 1993; Loeb, 1991).

MSI is observed in almost all adenocarcinomas from patients with hereditary non-polyposis colorectal cancer (HNPCC) (Aaltonen *et al.*, 1994 a, Aaltonen *et al.*, 1994 b; Konish *et al.*, 1996) and occurs in 10- 15 percent of sporadic colorectal cancers (Ionov *et al.*, 1993; Aaltonen *et al.*, 1994 a, Aaltonen *et al.*, 1994 b, Konish *et al.*, 1996, Thibodeau *et al.*, 1993, Jass *et al.*, 1998; Lothe *et al.*, 1993). HNPCC is an autosomal dominantly inherited condition that accounts for approximately 5% of all cases of colorectal cancer (Wheeler and Bodmer, 2000). It is characterized by relatively small numbers of adenomas, the development of colorectal cancer at an early age, a predominance of tumors in the proximal colon and is sometimes associated with extra colonic tumors, such as gastric and endometrial cancer (Wheeler and Bodmer, 2000; Markowitz and Winawer, 1997).The presence of MSI-H in sporadic colorectal cancer and HNPCC correlates significantly with a number of clinical and pathological features including proximal location (Ionov *et al.*, 1993; Aaltonen *et al.*, 1994 a; Thibodeau *et al.*, 1993), diploid DNA content (Ionov *et al.*, 1993; Lothe *et al.*, 1993; Thibodeau *et al.*, 1998) and a poor mucinous differentiation (Lothe *et al.*, 1993; Muta *et al.*, 1996).

To date, mutations in five human MMR genes have been described: hMSH2, hMLH1, hPMS1, hPMS2 and MSH6 (Wheeler and Bodmer, 2000). The majority of those with HNPCC have a germ line mutation in one theses genes with HNPCC have a germline mutation in one of these genes, with more than 90% of cases involving hMSH2 or hMLH1(Kinzler and Vogelstein, 1996). Furthermore, many HNPCC tumors harbor mutations in both alleles, one germline and one somatic, or total absence of wild type allele, indicating that both copies of an MMR gene are inactivated before a tumor develops (Papadopoulos *et al.*, 1994; Leach *et al.*, 1993).Somatic mutations of these genes are found less frequently in sporadic MSI cancers (Liu *et al.*, 1995; Borresen *et al.*, 1995), suggesting that novel genes may be involved. However, lack of hMLH1 or hMSH2 expression has been demonstrated in 95 per cent of sporadic MSI-H tumors (Thibodeau *et al.*, 1998) and loss of

hMLH1 expression is known to be associated with hypermethylation of the promoter region of this gene.

Of particular interest is the high frequency of TGF-β type II receptor (RII) mutations in MSI-positive colorectal tumors. The gene coding for this receptor contains mononucleotide repeats, analogous to microsatellites, and so is particularly sensitive to defects in DNA mismatch repair. Inactivating mutations of RII have been found in 90 per cent of colorectal cancers showing MSI, in most cases both alleles are affected (Parsons *et al.*, 1995). RII mutations have also been studied in MSI-positive adenomas at various stages of progression (Grady *et al.*, 1998).The earliest stage at which RII mutations were detectable was in high-grade dysplastic adenomas, and in adenomas containing a focus of invasive carcinoma the prevalence of RII mutations was approximately 75per cent. These observations suggest that a mutation of TGF-βRII is a critical step in MMR-deficient colorectal tumor formation that RII behaves like a tumor suppressor and that its mutation correlates strongly with the progression of adenoma to carcinoma. However, it is interesting that RII mutations are also common in MSI-positive gastric cancer but rare in MSI-positive endometrial cancer (Myeroff *et al.*, 1995).These findings suggest that RII mutations do not simply reflect MSI but are instead selected for and contribute to the genesis of gastrointestinal cancers.

2.4.3 Transforming growth factor- β (TGF- β) pathway

A more recently identified pathway in CRC carcinogenesis involves mutations along the TGF- β signaling cascade. TGF- β binds its cell surface receptor TGF- β RII with the subsequent activation of SMAD proteins. These proteins translocate to the nucleus and regulate genes involved in the inhibition of cellular proliferation (Takayama *et al.*, 2006). Several mutations in TGF-bRII render this receptor resistant to the effects of TGF-β. Approximately 20% to 30% of CRCs display mutations in the TGF-b signaling pathway. In up to 90% of cases these mutations also display MSI (Kitisin and Mishra, 2006; Grady *et al.*, 1999). Mutations also are identified in SMAD proteins that cause the TGF-b signaling pathway to become dysfunctional (Eppert *et al.*, 1996; Ando *et al.*, 2005). Direct interactions between SMAD proteins within the TGF-b pathway and signaling molecules in the Wnt pathway have been identified and it is suggested that these two pathways may work synergistically in promoting tumorogenesis (Labbe *et al.*, 2000). The mutations involving the TGF-b pathway seem to be a late occurring event in the adenoma to carcinoma progression.

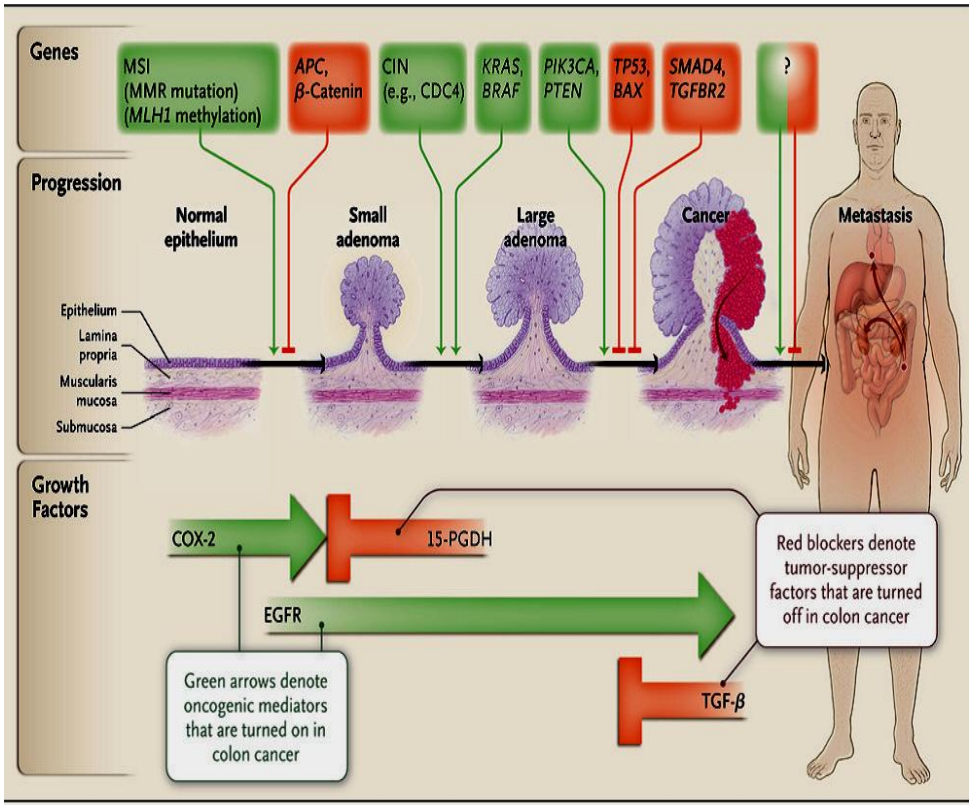

Figure .2: Genes and Growth Factor Pathways That Drive the Progression of Colorectal Cancer.

(Source: Molecular Basis of Colorectal Cancer. New England Journal of Medicine (2009). Vol. **25**, pg. 361

2.5 DNA repair of alkylated bases

Two main processes for the repair of alkylation damage to DNA have been described, one is a DNA glycosylase, which cleave the base-sugar bond of abnormal nucleotide residues and the other is a O^6-alkylguanine transferase. Remarkable features are the relatively high degree of specificity of these DNA repair processes, and the similarity that has been observed in these major repair pathways between *Escherichia coli* and mammalian cells, including those of human origin,particularly on the O^6-methylguanine-transferase (Lindahl, 1982).

The information on repair of O-alkylpyrimidines, although still rather limited, shows that: O^2-methylcytidine and O^2-methylthymine are repaired by the same enzyme (3-methyladenine DNA glycosylase II), which repairs 3-methyladenine and 3-methylguanine; in *Escherichia coli* at least a 2-fold greater release from DNA is observed with the O^2-methylpyrimidines as compared with the N^3-methylpurines (McCarthy *et al.*, 1984); O^4-methylthymine is repaired by the methyltransferase that also acts on O^6-methylguanine in DNA in *E. coli* (Laval & Laval, 1984). In mammalian cells and in *E. coli,* O^6-methylguanine transferase can also function on ethylated, chloroethylated and propylated DNA, although with a lower rate of removal (Robins *et al.*, 1983).

2.6 O^6-Methylguanine-DNA methyltransferase (MGMT)

Studies in the 1970s and 1980s examined the formation and persistence of O^6alkyl guanine in DNA originally in bacteria and rat liver and subsequently in other species. Studies on animals used carcinogenic dose regimes and in most cases, the formation, accumulation or persistence of this lesion in the DNA of specific tissues correlated with their susceptibility to carcinogenesis. This was attributed to the mutagenic properties of the base. The presence of O^6-methylguanine was first reported in 1985 and this and other alkylation damage products are increasingly evident in human DNA although the sources of such damage have yet to be identified (Povey, 2000).

The early studies showed that O^6-methylguanine was lost from DNA in rodent tissues more rapidly than could be accounted for by cell turnover and this indicated the existence of an active repair system (Lawley and Orr, 1970; O'Connor *et al.*, 1973). The fact that this could be saturated and subsequently recover, following pre-treatment with various DNA

damaging agents was the first suggestion of the novel mechanism of this repair system (Kleihues and Margison, 1976).The characterisation of the repair processes responsible occurred more or less simultaneously in prokaryotic and eukaryotic systems. The prokaryotic proteins were initially shown to act on O^6 MeG in methylated DNA and it was reasonable to refer to them as methyltransferases (hence *MGMT*). However, the term alkyltransferase is also frequently used and has the advantage that it avoids confusion with the DNA methyltransferase that generates 5-methylcytosine in DNA using S-adenosylmethionine as a methyl group donor. More importantly, *MGMT* can act on the minor product O^4methylthymine and clearly have a very broad specificity it is evident from work with alkylated DNA and low molecular weight pseudo-substrates that the human *MGMT* can act at different rates on a very wide range of O^6 alkyl groups (Pegg, 2000).

2.6.1 Occurrence and physiological effects of MGMT

O6-Alkylguanine-DNA alkyltransferase was first isolated from E. coli in the late 1970s as the Ada protein, a dual function inducible repair protein, which regulates the adaptive response to low levels of alkylating agents (Demple *et al.*, 1985; Foote *et al.*, 1980; Lindahl *et al.*, 1988; Olsson and Lindhal, 1980). E. coli has a second alkyltransferase, the constitutively expressed Ogt protein(Potter *et al.*, 1987).The human MGMT cDNA was first isolated from a cDNA library by rescue of the ada phenotype in E. coli.(Tano *et al.*, 1990).The characterization of MGMT and its homologues is an ongoing process. O6-Alkylguanine-DNA alkyltransferases are found in prokaryotes, archea bacteria and many eukaryotes, but not in the plant kingdom. The evolutionary conservation of MGMT suggests that it plays a fundamental role in maintaining genomic integrity. However, MGMT is not essential for cell viability. It has been shown for many cell types that sensitivity to alkylating agents inversely correlates with MGMT activity and that MGMT-deficient cells are more susceptible to spontaneous and alkylating-agent-induced toxicity and mutation. Furthermore, MGMT knockout mice are more susceptible to toxicity and tumor induction by alkylating agents, whereas mice over expressing MGMT are more resistant (Margison and Santibanez, 2002). Thus MGMT protects both normal cells and tumor cells against the toxic and mutagenic effects of O6-alkylating agents and is therefore a crucial factor in mediating the resistance to this class of chemotherapeutic agents.

2.6.2 MGMT Gene characterization and regulation

The gene for AGT, which is referred to as *MGMT*, has been characterized from both human, where it is present on chromosome 10q26, and mouse, where it is present near the telomere of chromosome 7 (Iwakuma *et al.*,1996; Pieper *et al.*,1990; Tano *et al.*, 1997). The organization of the gene is similar in both cases with five exons one non-coding and four introns, three of which are very large. The second intron in the mouse is >110 kb and three of the human introns each span >40 kb. Thus, *MGMT* is a very large gene (>170) kb. which encodes a mRNA of slightly less than 1 kb. A mouse 3T3 cell line selected for resistance to chloroethylating agents was found to have an amplification of the *MGMT* gene (Tano *et al.*, 1997). Such amplification appears to be a very rare phenomenon, which may be related to the size of the gene and to the means by which alkylating agents induce cell killing, being non-conducive to amplification mechanisms.

The promoter region of the *MGMT* gene has been characterized as a 1.2-kb region located at positions -954 to + 203 in the gene. It is very CpG-rich, lacks TATA and CAAT boxes and has 10 Sp1 transcription factor binding sites. These features are all consistent with the constitutive expression of AGT in all tissues. The promoter contains two possible glucocorticoid response elements and two AP-1 sites located 1 kb upstream of the transcription start site. The GREs are not a perfect match with the consensus sequences but direct proof that AGT expression may be regulated by glucocorticoids has been provided both by showing an increased AGT activity in HeLa S3 cells treated with dexamethasone and by showing that this steroid increases expression of a luciferase reporter gene under the control of the *MGMT* promoter (Biswas *et al.*, 1999). The changes were quite small with only a 2-fold increase in AGT activity and protein and a 3-fold increase in reporter gene expression. Nonetheless, there was a modest increase in resistance of the treated cells to BCNU. The possible importance of the AP-1 sites has been revealed by similar studies showing increased production of luciferase from the *MGMT* promoter-driven gene in cells transfected with c-*jun* and c-*fos* and that activators of protein kinase C such as TPA or DAG and inhibitors of protein phosphatases such as okadaic acid increase the level of AGT mRNA and increase resistance to BCNU in HeLa cells (Boldogh *et al.*, 1998). As with dexamethasone, the level of increase was small, in the range of 4-fold. A 59-bp DNA sequence located at the first exon-intron boundary has been shown to act as an enhancer needed for efficient expression of the mRNA. A very interesting protein that binds to a 9-mer sequence in this region has now been identified and was found to be in the nucleus of cells expressing AGT (Chen *et al.*, 1997). In cells that exhibit the Mer- phenotype and lack

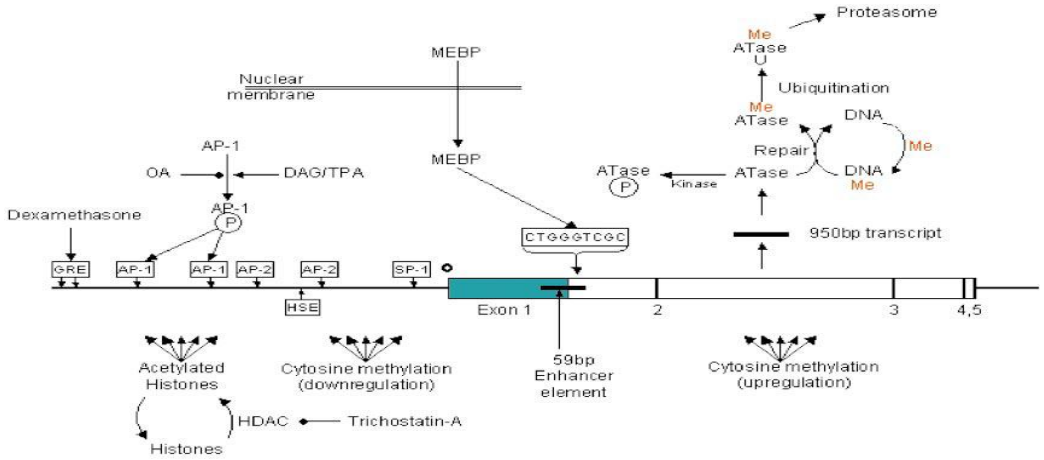

Figure 3: Structure of the human MGMT gene and factors known to affect the expression and activity of MGMT. The maximal promoter and exon 1 region is expanded to show more detail. The positions of exons 2-5 are shown as vertical bars. O indicates the origin of transcription.

(Source: Variability and regulation of O6-alkylguanine±DNA alkyltransferase Carcinogenesis (2003) vol.**24** pp.625-635

AGT expression, the enhancer binding protein was cytoplasmic. The factors responsible for the change in localization of this protein may therefore be of great importance in maintaining the level of AGT in mammalian cells. One possible way in which AGT levels might be regulated in response to DNA damage is through p53. p53 is known to interact with the transcription factor Sp1 and the *MGMT* promoter contains Sp1 sites. However, the findings are currently confusing and there may be both cellular and species variations in the effects as well as differential responses of basal and induced expression. Transfection of wild type but not mutant p53 blocked the *MGMT* promoter activity in Saos-2 cells and transduction with an adenoviral vector expressing p53 down regulated the AGT content of human fibroblasts (Harris *et al.,* 1996). Down regulation of the human *MGMT* promoter by p53 was also seen in rodent cell lines (Grombacher *et al.*, 1998). However, ionizing radiation is known to induce AGT and p53 (Rafferty *et al*., 1996) suggesting that any increase in p53 should be associated with a rise in AGT content. This would also make sense in terms of the generally accepted role of p53 in enhancing DNA repair. In support of this concept, it has been reported that ionizing radiation fails to induce AGT in p53 null mice and the response is ablated in mice with only one copy of the p53 gene (Rafferty *et al.*, 1996). Also, the expression of reporter genes under the control of the *MGMT* promoter is increased by ionizing radiation in rodent cells with wild type p53 but not with mutant p53, and only the cells with wild type p53 showed a radiation-induced increase in AGT activity (Grombacher *et al.,* 1998).

Although the basal level of AGT in the mouse tissues studied was unaffected by p53 status (Rafferty *et al.,* 1996), the level of AGT in astrocytes was much reduced in mice lacking p53 (Nutt, *et al.,* 1999). It is clear that much more work needs to be carried out on the role of p53 in regulating AGT levels in both the basal and induced states. Experimental protocols in which un-physiologically high (or low) levels of p53 are used may not provide relevant information and the extrapolation of results obtained with mice to human cells may not be straightforward. More careful examination of the interaction of p53 with components of the AGT transcription system is needed. In this regard, it should be noted that the inhibitory effect of p53 on basal AGT expression may not require the p53 DNA binding domain (Grombacher *et al.,* 1998).

2.6.3 Structure of MGMT Protein

Despite the extremely low primary sequence identity between human, *E. coli*, and the archae bacteria *Pyrococcus kodakaraensis* (Pk). AGT (14% identity, 26% similarity.), the

overall domain structure and fold are nearly identical between the three homologs (Moore *et al.,* 1994; Hashimoto *et al.,* 1999; Daniels *et al.,* 2000; Wibley *et al.,* 2000). AGT exhibits a two-domain α/β fold with overall dimensions of approximately 20×35×40 A°. While the three species have no significant primary homology in the N-terminal domain (approximately residues 1–85), they exhibit a conserved a/β roll. All three structures have a three-stranded anti-parallel β-sheet followed by two (human, bacterial.) or three (Pk) helices, with relatively small differences in the lengths and orientation of these secondary structures.

The topology of the C-terminal domain is absolutely conserved, being composed of a short two stranded parallel β-sheet, four a-helices, and a 3_{10} helix bearing the invariant active-site PCHR motif. The nucleophilic cysteine lies near the bottom of a solvent accessible groove roughly 7 A° wide, 9 A° deep and 14 A° long that defines an *O*6-alkylguanine binding channel. Immediately preceding the active-site motif are two overlapping, tight turns stabilized by an absolutely conserved asparagine (the Asn-hinge), and a helix-turn-helix (HTH) DNA-binding motif (Fig 2.5)

While the overall structures of AGT from the three kingdoms of life are remarkably conserved, some differences do exist. Human AGT contains a zinc-binding site that does not exist in the bacterial or hyperthermophilic structures. The Zn (II) Ion bridges three strands of the N-terminal b-sheet and the coil immediately preceding the domain-spanning helix via tetrahedral ligands (Cys5, Cys24, His29, and His85 (Daniels *et al.,* 2000). These ligands are absolutely conserved in the known mammalian sequences, implying that this binding site may be general to higher eukaryotes. The role of the zinc ion is likely structural, serving to stabilize the interface between the N- and C-terminal domains by tethering the domain-spanning helix to the three strands of the N-terminal β-sheet. Consequently, the His-tagged human structure (Wibley *et al.,* 2000), which lacks this Zn(II) ion, shows small conformational differences and increased disorder in the N-terminal domain (Daniels *et al.,* 2000). This slight structural relaxation is presumably due to the removal of Zn during purification over metal affinity columns. Loss of zinc stabilization may also account for approximately two-fold reduction in the apparent second-order rate constants observed for His-tagged relative to native recombinant AGT (Daniels *et al.,* 2000; Goodtzova *et al.,* 1998).

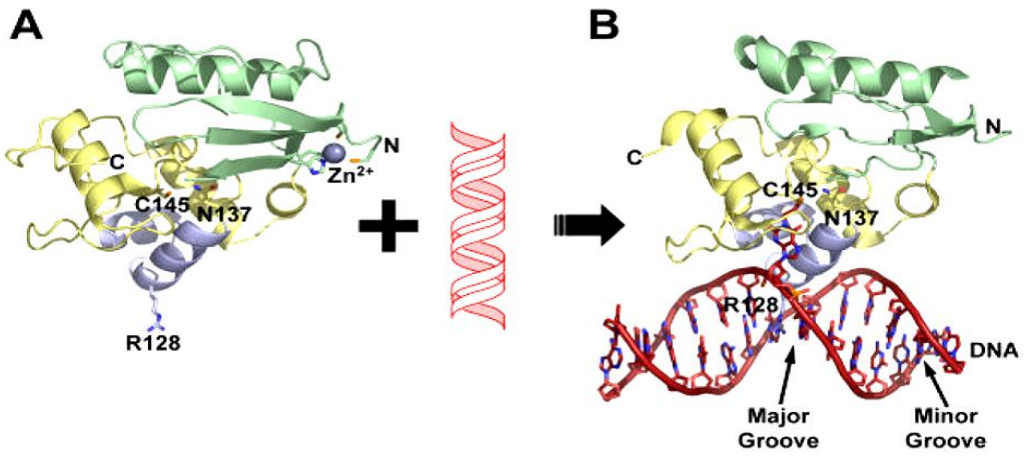

Figure 4: Human MGMT X-ray crystallographic structure, zinc site, and DNA binding. (A) Unreacted MGMT structure (pdb 1eh6). The N-terminal domain is shown in green, the C-terminal domain is shown in yellow, and the HTH motif is shown in blue. The active site cysteine, arginine finger, "Asn hinge", and zinc ligands are shown in ball-and-stick representation. (B) DNA-bound MGMT structure (pdb 1t39). MGMT uses the recognition helix of the HTH motif to bind the minor groove of DNA.

[Source: Tubbs, J.L., Pegg, A.E., Tainer, J.A. (2007). DNA binding, nucleotide flipping, and the helix-turn-helix motif in base repair by O6-alkylguanine-DNA alkyltransferase and its implications for cancer chemotherapy DNA Repair; **6**(8): 1100–1115].

2.6.4 Mechanism of MGMT action

The biochemistry of the repair reaction and the amino acid residues required have been extensively explored by site- directed mutagenesis (Crone *et al.*, 1994), selection of cDNAs from random libraries (Encell *et al.*, 1998) and the reaction with alkylated oligonucleotides and structurally diverse pseudo-substrates (Spratt *et al.*, 1999). The current model for the mechanism of MGMT is that it binds to DNA via a helix-turn-helix motif in the C-terminal domain. The second helix contains an arginine ``finger'' that flips out the alkylated base into the active site. A histidine residue within the highly conserved active site motif PCHRV/I and a glutamic acid residue form a hydrogen bond network with the active site cysteine residue affecting the transfer of the alkyl group to the latter. This stoichiometric reaction results in ``suicidal'' inactivation of MGMT and alters the conformation of its DNA-binding domain. The alkylated protein thus becomes detached from DNA and also targeted for degradation by ubiquitination (Srivenugopal *et al.*, 1996). The auto-inactivating mechanism means that the continued repair of O ^{6}-alkylation damage in DNA requires *de novo* synthesis of active protein. The active site pocket must be quite flexible since a wide range of different structures are recognised as substrates, in accord with the concept of ``induced fit''. MGMTs from different sources can act at different rates on the same pseudo-substrate, however, so that primary structural differences are also important. Modification of the amino acid sequence has been used to change substrate specificity (Encell *et al.*, 1998) in the development of protective gene therapy strategies.

MGMTs have no known cofactor requirements. Although evidence has been presented for the co-localisation of MGMT with nascent RNA (Ali *et al.*, 1998), the rate of removal of O^6MeG from transcribed and non-transcribed genes is similar (Engelbergs *et al.*, 1998). Phosphorylation of the protein, resulting in a decrease in activity, has recently been reported (Srivenugopal *et al.*, 2000).

One exception to the general alkyl transfer repair mechanism is the action of the protein on N1-O^6-ethenoguanine. This is formed after chloroethylation of the O^6-position of guanine and is an intermediate in DNA inter strand crosslink formation. Whilst the chloroethyl group can be removed by the alkyl transfer mechanism, in the case of N1-O^6-ethenoguanine, MGMT forms a covalent bond with the C atom next to the O ^{6} atom; but as the etheno group is attached to the N1 atom, the DNA becomes covalently attached to the protein (Gonzaga *et al.*, 1992). Whether or not this reaction occurs *in vivo* and, if so, the fate of the bound MGMT molecules and the biological significance of this, if any, remain to be established. There is some evidence that nucleotide excision repair may be involved in the

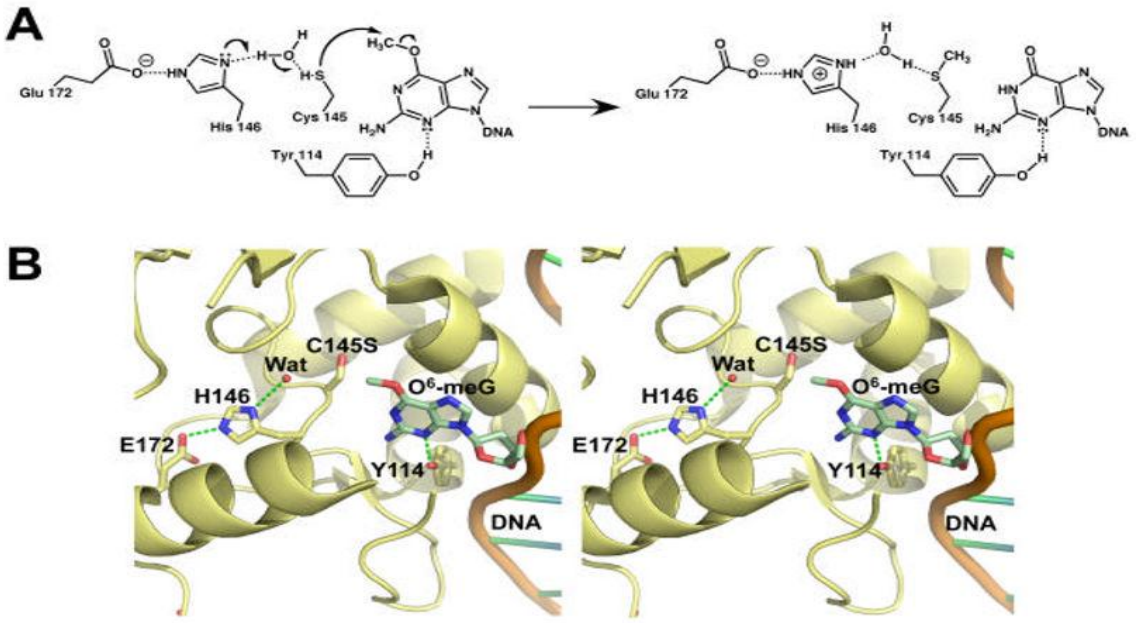

Figure 5: The AGT active site and proposed reaction mechanism. (A) Hydrogen bond network andproposed reaction mechanism for AGT. To facilitate attack at the *O6*-alkyl carbon, His146 actsas a water-mediated general base to deprotonate Cys145. The resultant imidazolium ion isstabilized by Glu172. Tyr114 donates a proton to N3 of *O6*-methylguanine. (B) Stereo viewof the X-ray crystallographic structure of the AGT C145S-DNA complex (pdb 1t38) showingthe active site, *O6*-methylguanine substrate, and hydrogen bond network.

[Source: Tubbs, J.L., Pegg, A.E., Tainer, J.A. (2007). DNA binding, nucleotide flipping, and the helix-turn-helix motif in base repair by O6-alkylguanine-DNA alkyltransferase and its implications for cancer chemotherapy DNA Repair; **6**(8): 1100–1115].

processing of such a lesion given that NER-defective cells are more susceptible to chloroethylating agent toxicity (Wu *et al.*, 1992).

Transfer of alkyl groups from the O^4-position of thymine in DNA by mammalian MGMT is inefficient and there is evidence that binding to such lesions can result in shielding of this from repair by the nucleotide excision repair pathway (Samson *et al.*, 1997). More recently, MGMT has been shown to protect cells against the toxic effects of cyclophosphamide, possibly via the repair of acrolein adducts in DNA (Friedman *et al.*, 1999).

2.6.5 MGMT hypermethylation and protein expression

Due to the important role of MGMT in DNA repair, several studies have been undertaken to find correlations between MGMT methylation status, expression, and tumorigenesis.

2.6.5.1 MGMT in non-tumorous tissues.

As a repair enzyme, MGMT is expressed in every human tissue, but its level in individual tissues varies, even in subpopulations. The levels and activity of MGMT in healthy cells are regulated by its protein phosphorylation status (Srivenugopal *et al.*, 2000), the binding of the E6 papilloma virus oncoprotein (Srivenugopal and Ali-Osman, 2002) and the action of p53 (Harris *et al.*, 1996), glucocorticoid hormone (Biswas *et al.*, 1999) and other transcription factors over its 50-CpG island, which includes a classical promoter without TATA and CAAT boxes and a 59 bp enhancer element located at the first exon–intron boundary (Harris *et al*,.1991). Its expression is highest in the liver, relatively high in lung, kidney and colon, and the lowest in pancreas, hematopoietic cells, lymphoid tissues and brain (Gerson, 2004; Sabharwal and Middleton, 2006; Kaina *et al.*, 2007; Liu and Gerson 2006; Soejima *et al.*, 2005; Pegg *et al.*, 2007; Helleday *et al.*, 2008; Pieper, 1997). Furthermore, MGMT expression levels in tissues remain stable with age (Bobola *et al.*, 2007). The study of Bobola examined MGMT activity in 71 brains at 6 to 19 weeks of gestation and found activity in such developing brains to be directly correlated with gestational age and that the proportion of specimens negative for MGMT at 19 weeks was approximately that observed in adults. Prior to 19 weeks of age, fetal brain levels of MGMT were low, suggesting that exposure to exogenous and endogenous alkylators prior to that time may, due to vascular permeability, such as the blood-brain barrier, result in increased susceptibility to tumor formation in later life (Bobola *et al.*,2007).

2.6.5.2 MGMT in tumor tissues

Several studies have found that MGMT protein is decreased in some tumors with respect to their normal tissue counterpart (Gerson *et al.*, 1986; Citron *et al.*, 1992) .In fact a subset of tumor cell lines, termed Mer-, completely lack MGMT activity (Day *et al.*, 1980). For example, Silber assessed levels of MGMT in normal brain tissue and in primary brain tumors (Silber *et al.*, 1998). They found relatively but significantly lower amounts of MGMT in tumors (Bobola *et al.* 1996). Also of interest were the observations of Sawhney *et al.* who found significant loss of MGMT expression in the transition of hyperplasia to dysplasia in patients with oral squamous cell carcinoma (Sawhney *et al.*, 2007). This suggested that diminution in MGMT expression is an early step in oral tumorigenesis. As loss of expression is not commonly due to deletion, mutation or rearrangement of the MGMT gene (Day *et al.*, 1980; Fornace *et al.*, 1990; Pieper *et al.*, 1990) or mRNA instability (Kroes and Errickson, 1995), other causes for loss of activity are expected. Methylation of cytosine in CpG dinucleotides is the main epigenetic modification of DNA in normal mammalian cells (Esteller and Herman, 2002). The human MGMT gene possesses a CpG island in the 5' portion of the gene (Harris *et al.*, 1991). The region from −249 to +259 at the transcription start site is critical in DNA methylation-associated silencing (Qian and Brent, 1997). Hypermethylation of normally unmethylated CpG islands in the promoter regions of many genes, including p16INK4a, p14ARF, VHL, BRCA1, hMLH1 and E-cadherin, correlates with its loss of transcription (Esteller *et al.*, 2001a, Esteller, 2002b). Hypermethylation of the MGMT CpG Island as the cause of MGMT transcriptional silencing in cell lines defective in O6-methylguanine repair has been demonstrated (Costello *et al.*, 1996; Qian and Brent., 1997; Watts *et al.*, 1997; Danam *et al.*, 1999; Esteller *et al.*, 1999a). As for other genes inactivated by CpG island methylation, hypermethylation of the promoter region is accompanied by histone hypoacetylation and methylation (Kondo *et al.*, 2003), binding of specific methyl-binding proteins (Fraga and Esteller, 2002) and loss of nucleosome positioning (Patel *et al.*, 1997), all of these alterations rendering a 'closed' chromatin state that prevents gene transcription (Ballestar and Esteller, 2002). As additional proof of causality, in vitro treatment of cancer cells with demethylating drugs restores MGMT expression (Qian and Brent, 1997; Esteller *et al.*, 2000 a). To study the relevance of the promoter hypermethylation of the MGMT gene in vivo in cancer patients, Estellar examined a large series of more than 500 primary human tumors and corresponding normal tissues for MGMT aberrant methylation using methylation-specific PCR and its relation with MGMT expression (Esteller *et al.*, 1999 a). MGMT

function is lost frequently in association with hypermethylation of the promoter region in a wide spectrum of human tumors (Esteller *et al.*, 1999 a). Their initial findings demonstrated a specific profile of MGMT hypermethylation in human cancer that indicated gliomas, lymphomas, colon, head and neck and non-small-cell lung carcinomas as the main tumor targets for the epigenetic inactivation of MGMT (Esteller *et al.*, 1999a). This profile, has been expanded in additional reports of MGMT inactivation in other tumor types (Toyooka *et al.*, 2001; Virmani *et al.*, 2001; Choy *et al.*, 2002; Lee *et al.*, 2002; Smith-Sorensen *et al.*, 2002). This aberrant MGMT methylationhas been correlated with the loss of MGMT protein (Esteller *et al.*, 1999 a; Esteller and Hermann, 2002; Herfarth *et al.*, 1999.), lack of mRNA expression (Esteller *et al.*, 2000 a; Yin *et al.*, 2003) and loss of enzymatic activity (Herfarth *et al.*, 1999).

The constellation of tumor types where MGMT hypermethylation can be invoked has also been further understood with its analysis in a comprehensive panel of 70 human cancer cell lines (Paz *et al.*, 2003). Furthermore, the CpG island hypermethylation-associated silencing of MGMT occurs very early in human tumorigenesis, such as in small colon adenomas (Esteller and Hermann, 2000), strongly supporting its relevant role in carcinogenesis. MGMT promoter hypermethylation has also been demonstrated in the serum DNA of lung cancer patients (Esteller *et al.*, 1999 b), and head and neck cancer patients (Sanchez-Cespedes *et al.*, 2000) as well as other biological fluids, including sputum (Palmisano *et al.*, 2000) and saliva (Rosas *et al.*, 2001). Capper evaluated MGMT expression as a prognostic factor in diffuse astrocytic tumors (n=162) ranging from low to high grade. The study found MGMT expression to be less prominent as the tumors became more malignant (Capper *et al.*, 2008). In other words, high-grade gliomas showed less MGMT expression than low-grade gliomas, suggesting a loss of MGMT expression in the process of anaplastic transformation (Capper *et al.*, 2008). Thus, it may be possible to use MGMT expression as a prognostic indicator, MGMT levels being a reflection of tumor grade. In colorectal cancer, Zhang reported longer survival rates in patients with MGMT negative surgical margins; however methylation did not have a significant effect upon overall survival in the 24-case series of colorectal cancer (Zhang *et al.*, 2007). Thus, it appears that methylation of the *MGMT* promoter region is an important predictor of patient survival in various tumor types.

2.6.6 MGMT and protection against point mutations

The transcriptional silencing of MGMT by promoter hypermethylation instigates an important mutator pathway in human cancer, because the *O*6-methylguanine adducts,

resulting from alkylating agents are not removed, thereby producing G:C to A:T transitions(Fig.2.7). Supporting these data, the most common mutations caused by alkylating agents are G:C to A: T transitions (Horsfall *et al.*,1990), exemplified in the frequent generation of G to A transitions in the oncogene K-ras when the carcinogen N-methylnitrosourea (that forms O6-methylguanine adducts) is used in experimentally induced tumor systems (Sukumar *et al.*, 1983; Mitra *et al.*, 1989). Avoidance of the mutagenic effect is directly related to the presence of a functional MGMT protein (Pegg *et al.*, 1995). In vitro assays show that endogenous MGMT expression protects mammalian cell lines from spontaneous G:C to A: T transitions in the aprt gene(Aquilina *et al.*, 1992). Animal models also show that transgenic mice over expressing MGMT are protected against O6-methylguanine-DNA adducts caused by methyl-nitrosourea (Dumenco *et al.*, 1993) and against G to A mutations in K-ras in aberrant colorectal crypt fociand lung tumors (Zaidi *et al.*, 1995; Liu *et al.*, 1999).This link between MGMT inactivation and K-ras transition mutations in human tumors was first observed in 2000 (Esteller *et al.*, 2000).The incidence of K-ras activation varies widely among carcinomas. K-ras mutation is rare in human primary breast carcinomas, but occurs in approximately half of colorectal carcinomas. This mutation distribution strongly resembles the pattern of MGMT promoter hypermethylation described (Esteller *et al.*, 1999 a), while MGMT aberrant methylation is not present in breast carcinomas where K-ras mutations are extremely rare, it occurs in approximately 40% of cases of colorectal carcinomas associated with loss of MGMT expression, and is also frequent in non-small-cell lung carcinoma (Esteller *et al.*, 1999b) where K-ras mutations are frequent. MGMT promoter hypermethylation was an early event in human colorectal tumorigenesis linked to the appearance of G to A mutations in the K-ras oncogene (Esteller *et al.*, 2000).The association between MGMT methylation and K-ras mutations has now been reported not only in colon cancer in (Whitehall, 2001) but also in gastric and gall bladder cancers (Park *et al.*, 2001; Kohya *et al.*,2003).The tumor suppressor p53 is the most commonly mutated gene in human cancer, and transition mutations constitute the most common p53 mutations (Greenblatt *et al.*, 1994; Pfeifer, 2000). Approximately 52% of the mutational events are mis-sense transitional changes, and, of this subset, 72% are G:C to A: T transitions (Greenblatt *et al.*, 1994). The profile of the mutational spectrum varies according to tumor type. Lung and head and neck tumors of smokers have a higher number of transversions, whereas colorectal tumors have the highest rate of transition mutations, reaching 70% of the total number of p53 mutations (Greenblatt *et al.*, 1994). These last mutations occur frequently in CpG dinucleotides, which are

normally methylated (Pfeifer, 2000; Rideout *et al.*, 1990; Tornaletti and Pfeifer, 1995), through increased rates of spontaneous deamination at methylcytosine, although other mechanisms are also conceivable. However, 17% of p53 mutations are transition mutations in non-CpG dinucleotides, where this causality cannot be invoked (Greenblatt *et al.*, 1994). Thus, G:C to A: T changes in p53 in non-CpG and CpG dinucleotides could be attributable, in part, to a defect in MGMT that allows the persistence of O6-methylguanine and its reading as an adenine (Fig 2.7).Promoter hypermethylation of MGMT was strongly linked to the presence of G:C to A: T transition mutations in p53, particularly in non-CpG dinucleotides (Esteller *et al.,* 2001) in colon cancer and has also been found in glioma (Nakamura *et al.*, 2001; Yin *et al.*, 2003), liver (Zhang *et al.*, 2003) and non-small-cell lung carcinomas (Wolf *et al.*, 2001). Yet to be classified is the particular source of promutagenic adducts in the O6 position of guanine not repaired by MGMT in each tumor type. For colorectal tumors, the alkylating agents causing the pro-mutagenic lesion may be provided from dietary nitrates reduced in the proximal colon by bacteria, by nitrosation of amines and amides derived from protein catabolism (Ward *et al.*, 1989; Bartsch *et al.*, 1990; Rowland *et al.*, 1991). For other cancers, these carcinogens are ever more speculative. Not all adducts are repaired with the same efficacy. O6-ethylguanine is removed faster than the O6-methylguanine, perhaps preventing ras mutations in certain tumor types more exposed to these xenobiotics (Engelberg *et al.*, 1998). Among the glial tumors, MGMT epigenetic inactivation (Esteller *et al.*, 1999a) and MGMT loss or reduced activity (Hongeng *et al.*, 1997; Silber *et al.*, 1998) are common features, and these epigenetic events associate with the presence of transition mutation in p53 (Nakamura *et al.*, 2001; Yin *et al.*, 2003). Yet, carcinogens exposure is not frequently associated with the development of brain tumors.

2.6.7 MGMT and protection against carcinogenesis

The first report to conclusively show that DNA repair by AGT prevents cancer used the MNU-induced thymic lymphoma mouse model. Human *MGMT* thymic over expression in these mice completely suppressed the development of lymphoma (Dumenco *et al.,* 1993) under conditions in which the *O*6-MG adducts were efficiently removed (Liu *et al.*, 1994). Other transgenic mouse models with tissue-targeted *MGMT* expression had a reduction in liver tumours after treatment with dimethyl nitrosamine (Nakatsuru *et al.*,1993),two-stage skin tumours induced by MNNG (Kaina, 2004)and lung tumours induced by NNK (Liu *et al.*, 1999).When MGMT was over expressed in C3H mice, which normally

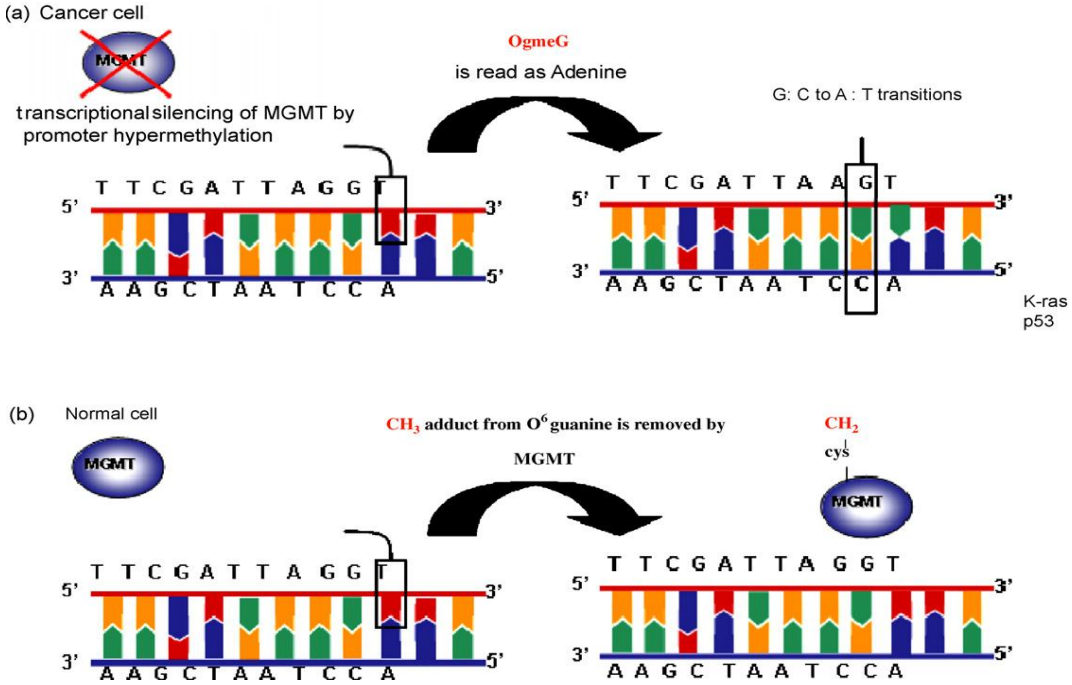

Figure 6: The promoter hypermethylation of MGMT in a wide spectrum of human tumours uncovers a new mutator pathway, because the O6-methylguanine adducts resulting from alkylating agents are not removed and this consequently causes G:C to A:T transitions that affect genes required for genomic stability.

have a high frequency of spontaneous liver tumours ,liver tumour formation was reduced, indicating that endogenous *O*6-MG lesions might be responsible for these tumours in susceptible mice (Zhou *et al.*,2001).Furthermore, in mice with other DNA-repair defects, such as MMR deficiency, transgenic *MGMT* over expression protected from MNU-induced carcinogenesis as well (Qin *et al.*, 1999).Mice predisposed to develop lymphomas because of over expression of the LIM domain oncogene-1 (*LMO1*)(Qin *et al.*, 1997) or loss of p53 are also protected from methylating-agent-induced lymphomagenesis by *MGMT* over expression (Reese *et al.*, 2001). As expected, *Mgmt*-knockout mice (*Mgmt*–) are more sensitive to methylating and chloroethylating agents than wild-type mice (*Mgmt*+). Although *Mgmt*-knockout mice are killed by low doses of MNU and BCNU (Glassner *et al.*, 1999), they can be protected by transplantation of normal marrow cells. In competitive repopulation assays, mixing wild-type with *Mgmt*-knockout marrow cells followed by transplantation and treatment with MNU results in a strong survival advantage in favor of the wild-type cells(Reese *et al.*, 2001). Furthermore, these mice survive doses of MNU that kill the knockout mice. This indicates that in the absence of *Mgmt*, marrow sensitivity, rather than other organ toxicity is dose limiting for chemotherapeutic alkylating agents (Reese *et al.*, 2001). Perhaps surprisingly, double knockouts of *Mgmt* and the MMR protein Mlh1 are highly sensitive to mutagenesis, but are resistant to drug-induced cell death (Kawate *et al.*, 1998).This is because without MMR proteins there is no recognition of persistent *O*6-MG DNA adducts.

2.6.8 MGMT Polymorphisms

There is an increasing realization that polymorphisms in genes involved in either the metabolism of carcinogens/toxins or the responses to damage caused by these agents including DNA repair may have a profound effect on sensitivity to these agents and thus on human health. Identification of polymorphic variants in such genes and understanding the mechanistic implications of such alterations is now a major research area. A large number of polymorphisms in the MGMT locus have been described. These slightly altered forms of MGMT may be useful as diagnostic tools, pre-treatment factors and indicators of risk of having a tumor. Some of the forms act as pre-treatment assessment tools as they are less sensitive to the action of certain MGMT inhibitors (Pegg *et al.*, 2007; Bugni *et al.*, 2007). In addition, they do have risk associations in the setting of breast, lung, head and neck,

colorectal and other types of cancer (Pegg et *al.*, 2007; Bugni *et al.*, 2007; Ogino et *al.*, 2007; Povey *et al.*, 2007). To date, 438 polymorphisms have been found (Povey *et al.*, 2007). They showed minor allele frequencies, defined as the relative abundance of a particular allele type among all individuals of a population, usually over 0.05% , and most affecting 5'-UTR, 3'-UTR and introns (Pegg *et al.*, 2007; Bugni et al .,2007; Povey *et al.*, 2007). The most common variants of MGMT are Ile143Val and Lys178Arg. Existing in nearly perfect disequilibrium with one another, they comprise 20% of observed variations (Bugni *et al.*, 2007). Other known variations affecting the primary structure of MGMT include Trp65Cys, Leu84Phe,Gly160Arg, Gly135Thr, Gly290Ala, Cys485Ala, Cys575Ala, Gly666Ala, Cys777Ala, Gly795Cys, Ala1034Gly and Cys1099Thr (Pegg *et al.* 2007). Both Leu84Phe and Ile143Val are of interest as they are situated close to the Cys145 active site and may affect MGMT function (Pegg *et al.*, 2007; Bugni *et al.*, 2007). . Most studies have focused on three common coding sequence polymorphisms - Leu84Phe (SNP ID: rs12917) , Ile143Val (rs2308321) and Lys178Arg (rs2308327) .

2.6.8.1 *Ile143Val*

Ile143Val, a polymorphism found in 18% of individuals of European origin, is not present in Nigerian and Japanese descendants (Povey *et al.*, 2007). The allele frequency is approximately 0.15% in Caucasians and is only rarely found in Asians and Africans (Bugni *et al.*, 2007). Located in a region of high linkage disequilibrium with 19 other polymorphisms, it has no effect upon pancreatic, prostate, oral and gastric cancer nor on melanoma risk, but is associated with a reduced risk in women for colorectal as well as head and neck cancer (Pegg *et al.*, 2007; Bugni et *al.*, 2007). Its correlation with lung, breast and endometrial cancer is debated (Pegg *et al.*; 2007; Bugni *et al.*, 2007). Some studies suggest it has no effect upon lung cancer (Krzesniak *et al.*, 2004; Yang *et al.*, 2004; Huang et al; 2005), whereas another found it to be weakly associated with an increased risk (Cohet *et al.*, 2004). Yet a third study reported a two-fold increased risk of lung cancer, particularly adenocarcinoma, in Caucasians and African-Americans with the Ile143Val (Kaur *et al.*, 2000). Similarly, one study noted that this variation had no effect upon breast cancer (Han *et al.* 2006), while another suggested that fruit and vegetable consumption resulted in a reduced breast cancer risk in women (Shen *et al.*, 2005). A recent study suggested that there is a reduced risk of endometrial cancer in smokers with the Ile143Val polymorphism. Interestingly, the same study found that Ile143Val carriers who had been smoking more than 30 pack-years had a significantly lower risk for endometrial cancer as compared to

non-smoking women homozygous for Ile143Val (Han *et al.*, 2006). The Ile143Val polymorphism results in MGMT equally capable of repair as is wild-type MGMT (Bugni *et al.*, 2007). In fact, after being incubated with *O6*-(4-bromothenyl) guanine (Patrin- 2, an MGMT inhibitor), it has been shown to be 1.3 times more active in repairing *O6*-MG adducts than is the wild type (Bugni *et al.*, 2007). It is also able to repair *O6*-[4-oxo-4-(3-pyridyl) butyl] guanine (*O6*-pobG) more effectively than both the wild-type and the Leu84Phe polymorphic MGMT (Pegg *et al.*, 2007). Ile143Val MGMT is less sensitive to *O4*-benzylfolate (BF), a powerfulMGMT inhibitor and slightly less sensitive to Patrin-2, the MGMT inhibitor mentioned above (Pegg *et al.*, 2007). The Ile143Val variant has reduced activity towards low molecular weight inhibitors, such as *O6*-BG and Patrin-2 (Pegg *et al.*, 2007). Ile143Val tumors respond poorly to chemotherapy (Bugni *et al.*, 2007). The role of Ile143Val expression is of importance as a pre-treatment marker since patients with this phenotype express reduced sensitivity to MGMT inhibitors, a factor that may affect treatment.

2.6.8.2 *Lys178Arg*

This polymorphism has no effect upon the risk of developing breast, pancreatic, prostate and gastric carcinoma or melanoma (Pegg *et al.*, 2007; Krzesniak *et al.*, 2004; Huang *et al.*, 2005; Han *et al.*, 2006; Shen *et al.*, 2005; Ma *et al.*,2003; Ritchey *et al.*,2005; Jiao *et al.* 2006). Some studies showed a weak association between Lys178Arg and increased lung cancer risk (Pegg *et al.*, 2007; Cohet *et al.*, 2004; Kaur *et al.*, 2000), whereas another study showed it to be associated with a lower lung cancer risk (Povey *et al.*, 2007). Lys178Arg is associated with reduced risk of colorectal, as well as head and neck cancer in women, and endometrial cancer in heavy smokers (Pegg *et al.*, 2007; Bugni *et al.*, 2007; Huang *et al.*, 2005). This polymorphism repairs *O6*-[4-oxo-4-(3 pyridyl) butyl] guanine (*O6*-pobG) more effectively than does either the wild-type or the Leu84Phe polymorphism MGMT (Pegg *et al.*, 2007). The Lys178Arg variant is less sensitive to the inhibitor BF and slightly less sensitive to Patrin-2 (Pegg *et al.*, 2007). Thus, Lys178Arg is also linked to an overall poorer response to chemotherapy (Pegg *et al.* 2007). As it is genetically related to the Ile143Val variant (Pegg *et al.*, 2007; Bugni *et al.*, 2007), there are studies on the association of the two forms. One found that the Ile143Val and Lys178Arg polymorphism carriers are better protected against the mutagenic effects of alkylating agents in comparison to wild-type carriers (Pegg *et al.*, 2007). As a prognostic factor, Lys178Arg expression appears to be useful in differentiating between patients in whom chemotherapy and MGMT inhibitor

therapy would or may not be successful, since this polymorphism is less sensitive to MGMT inhibitors and is associated with a poorer chemotherapeutic response.

2.6.8.3 *Leu84Phe*

The Leu84Phe variation has an allele frequency of 0.15% (Bugni *et al.*, 2007). Its expression does not vary significantly by race, but it is somewhat more prevalent among Caucasians than Chinese (Pegg *et al.*, 2007). One study demonstrated a correlation between this polymorphism and cancer risk, alcohol intake, body mass index (BMI) and post-menopausal hormone (PMH) use (Tranah *et al.*, 2006).This polymorphism has no effect upon lung, oral and gastric cancer or melanoma risk (Krzesniak *et al.*, 2004). It is, however, associated withan increased risk of breast cancer in heavy smokers (Shen *et al.*, 2005), an increased incidence of glioblastoma multiforme (Inoue *et al.*, 2003) as well as an increased risk of prostate (Ritchey *et al.*, 2005) and bladder carcinoma (Li *et al.*, 2005). Leu84Phe polymorphism carriers have a lower risk of endometrial cancer (Han *et al.*, 2006), a lower incidence of head and neck cancer (Huang *et al.*, 2005) and colorectal cancer associated with a better prognosis (Pegg *et al.*, 2007; Moreno *et al.*, 2006). Furthermore, women who consumed more than 0.5 alcoholic beverages per day with the Leu84Phe polymorphism had an increased risk of colorectal cancer, while those with the allele who drank less had a lower risk (Tranah *et al.*, 2006). In addition, women with the polymorphism and a body mass index (BMI) ≥25 had a lower risk of colorectal cancer than women with a BMI <25 who were homozygous for the wild-type allele (Tranah *et al.*, 2006). In those homozygous for the wild-type allele, PMH use was inversely proportional to colorectal cancer risk (Tranah *et al.*, 2006). Interestingly, no such correlation was noted in carriers of the Leu84Phe polymorphism. The same study found no association between colorectal cancer, polymorphism, BMI, and environmental factors including smoking and alcohol intake (Tranah *et al.*, 2006). Leu84Phe is as capable as wild-type MGMT in the repair of *O6*-MG adducts (Pegg *et al.*, 2007). One study found Leu84Phe variant proteins to be more susceptible to NNK-induced aberrations than the wild type (Bugni *et al.*, 2007). It was also suggested that the Leu84Phe polymorphism may affect Zn2+ binding to MGMT, which is known to enhance MGMT activity (Bugni *et al.*, 2007). Although the function of this polymorphism is very similar to that of the wild type, its expression may be of prognostic utility in heavy smokers, since NNK is primarily found in tobacco smoke (Pegg *et al.*, 2007; Bugni *et al.*, 2007).

2.6.9 Therapeutic manipulation of MGMT gene

Given its presence in human DNA and its mutagenic, toxic and recombinogenic potential, it seems unavoidable to conclude that O6-alk G can potentially be responsible for human cancer. However, whilst environmental alkylating agents have been extensively studied, the contribution of such exposures to cancer in the general population is not known. An understanding of the mechanisms of endogenous formation of this lesion and indeed of any exogenous agents that might enhance endogenous formation will help to define the relative contributions of exogenous and endogenous damage. This may lead to the identification of strategies that will reduce the levels of O6-alk G or its biological impact. It is also possible that further exploration of the factors affecting ATase expression might eventually allow modulation of ATase levels by means of exogenous agents, perhaps even in a tissue-specific manner. However, this does not appear to be a practical proposition at the present time. In terms of cancer therapy, agents that decrease ATase activity in tumours can make them more sensitive to the toxic effects of O6-alkylating agents, at least in experimental models. This is a strategy that is currently being pursued in cancer patients (Gerson, 2002): low molecular weight pseudosubstrates (O^6-BG, O^6-BTG) of ATase are in clinical trial (Spiro *et al.,* 1999; Schilsky *et al.*, 2000; Friedman *et al.,* 1998), and other approaches, including antisense and genetic suppressor elements are being explored in model systems. However, unless these strategies can be directed towards tumour cells, normal tissues are also likely to be sensitized to the toxic side effects of such therapies. Indeed this is the case in the trials thus far reported: it has been necessary to reduce the dose of the alkylating agents because of increased myelo-suppression (Roth *et al.*, 2000). Normal cell specific up-regulation may be an approach to circumvent this, but as already stated, it is not yet known how to effect this using exogenous agents. However, in the case of myelo-suppression, ex vivo haemopoietic stem cell transduction with retrovirus harboring ATase is being actively pursued (Roth *et al.*, 2000; Jelinek *et al.,* 1999). This approach could be used in the absence of repair inhibitors as it would be expected to protect against bone marrow toxicity and even allow O6-alkylating agent dose escalation. Nevertheless, current strategies are exploiting mutant versions of ATase that are resistant to inactivation by pseudo-substrates (Pegg *et al.*, 2000; Encell *et al.*, 1998) so that tumour sensitization and normal tissue protection can be achieved simultaneously by the use of inactivating agents.

The study was a case control undertaken to understand the etiology of colorectal cancer in Kashmir. Subjects with histopathologically confirmed colorectal carcinoma were evaluated. The colorectal carcinoma samples were collected from the Department of Surgery, Government S.M.H.S Hospital, 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. 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 was obtained. Record was maintained of the complete case history of the patients. The various methods that were used to analyse the epigenetic silencing of DNA repair gene *MGMT* by its promoter hypermethylation at CpG islands as a part of this study are described under.

Analytical reagents like absolute alcohol (ethanol), agarose, ethidium bromide, EDTA, TRIS acid and base, sucrose etc were procured from standard companies of high repute like Galaxo Laboratories, Merck, Qualigens, Sigma Chemical Company etc. similarly biomolecules like Taq DNA polymerase, proteinase K, dNTP's, primers etc. were also obtained from standard companies like Fermentas, Biotools, Cinnagen etc. Kits used in the study were from Zymo Research Ltd. List of chemicals used along with their manufacturers is given in Appendix.

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 histopatholgically 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 sample 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.

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^{TM}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 of **Genomic Lysis Buffer** (provided in the kit).
- The lyaste was centrifuged at 10,000 g for 5 min. and the supernatant was transferred to **Zymo-Spin^{TM}Column** in a Collection Tube and again centrifuged at 10,000 g for 1 min.
- Flow through along with the Collection tube was discarded and the **Zymo-Spin^{TM} Column** transferred to a new Collection tube and 200 µl of **DNA Pre-Wash Buffer** (provided in the kit) was added to the tube and centrifuged at 10,000 g for 1 min., this was followed by the addition of 500 µl of **g- DNA Wash Buffer** to the **Zymo-Spin^{TM} Column** and it was again centrifuged at 10,000 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^{0}C for a short time but the vials kept at -20^{o}C for longer duration storage for further investigation.

3.4.2 QUALITATIVE AND QUANTITATIVE ANALYSIS OF GENOMIC DNA

The integrity of the genomic DNA was examined by gel electrophoresis using 1% agarose gel to which 10µl / 50ml (of gel solution) of fluorescent dye ethidium bromide was added during its cooling and then gel was cast and 20µl wells were cast into it by usage of suitable combs. 2µl of each DNA sample was mixed with 1µl of 1-X DNA loading dye (1-X loading dye consists of 4.16 mg bromophenol blue,4.16 mg xylene cyanol and 0.66g sucrose in 1 ml water) and was loaded onto the gel. Electric current was applied at 50 volt until DNA entered in to the gel and was raised to 70 volt for rest of the run. Run was stopped when the dye had travelled nearly 2/3rd of the gel. DNA in the gel was visualized with the help of Gel doc system (Alphaimager TM 2200, Alpha Innotech Corporation) under UV light and picture was captured by using CCD camera system (Figure 7).

3.4.2.1 Quantitative Analysis

The quantity of the DNA was determined by measuring optical density (OD) 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 absorbance unit equates to 50µg/ cm^3 and therefore, the concentration of DNA sample is given by the following equation

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

The ratio of 260/280 nm was calculated and the DNA samples for which the ratio was 1.7-1.9 was 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^oC freezer for longer duration of time.

3.5 DNA MODIFICATION (BISULFITE TREATMENT)

Sodium bisulfite conversion will convert unmethylated cytosines to uracil and hence we are able to distinguish between the hypermethylated and non hypermethylated cytosine residues. DNA was modified by kit based method, the kit used was **EZ DNA**

Methylation™ Kit supplied by **ZYMO RESEARCH**. The kit has > 99% conversion efficiency of converting non-methylated Cytosines residues into Uracil, and the modified DNA recovery from the kit is > 80%. In addition the methylation kit shares innovative in-column desulphonation technology that eliminates otherwise cumbersome DNA precipitation steps while ensuring consistent results every time. The kit minimizes template degradation, loss of DNA during treatment and clean-up, and to provide complete conversion of unmethylated cytosines. Recovered DNA is ideal for PCR amplification. The kit protocol was followed.

- 500-1000ng of above isolated DNA samples were taken in different Eppendorf tubes.
- 5 µl of **M-Dilution Buffer** (provided in the kit) was added to each sample and final volume was made to 50 µl with distilled water.
- 100µl **CT-Conversion Reagent** (provided in the kit) was added to each tube and all tubes were placed 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 the **Zymo-Spin™ IC Column** (provided in the kit) and the column was placed into a provided **Collection Tube**.
- Sample from step 4 was loaded into the **Zymo-Spin™ IC Column** containing **M-binding buffer**. Cap was closed and mixing done by inverting the column several times.
- Centrifuge at full speed (≥10,000 g) for 30 seconds. Flow-through discarded.
- 100 µl of **M-Wash Buffer** (provided in the kit) was added to the column followed by centrifugation at full speed for 30 seconds.
- 200 µl of **M-Desulphonation Buffer** (provided in the kit) was added to the column and it was allowed to stand at room temperature ($20^{0}C$-$30^{0}C$) for 15-20 minutes. After the incubation, it was centrifuged at full speed for 30 seconds.
- 200 µl of **M-Wash Buffer** was added to the column, and centrifuged at full speed for 30 seconds; another 200 µl of **M-Wash Buffer** was added and again centrifuged for additional 30 seconds.

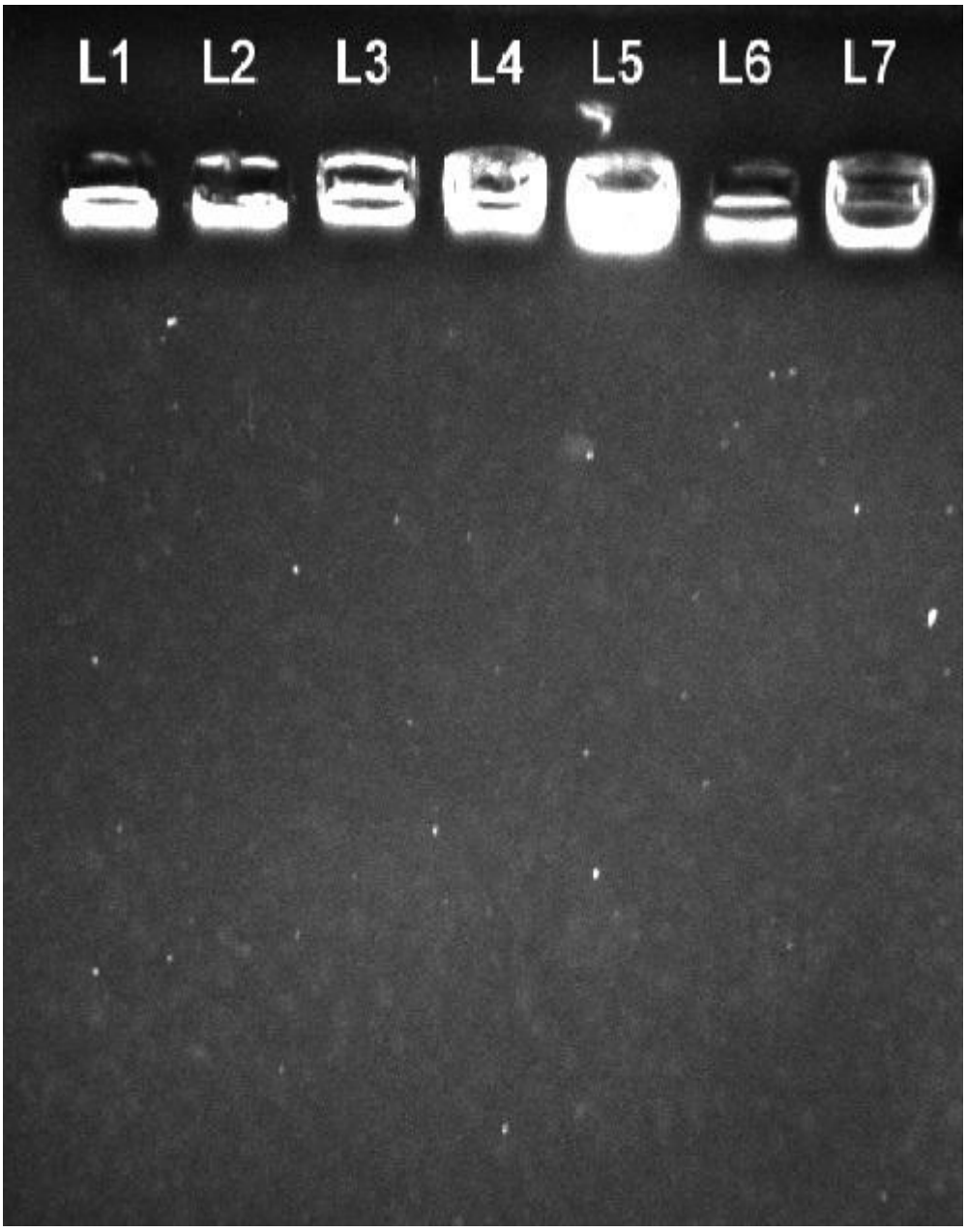

Figure 7: Representative gel picture showing the integrity of the genomic DNA on 1.0% agarose. Lane 1 to 7 contains the genomic DNA isolated from the tissue samples of colorectal cancer cases.

- Column was placed into a 1.5 ml micro centrifuge tube and of 15µl of **M-Elution Buffer** (provided in the kit) was directly added to the column matrix, and then it was centrifuged for 30 seconds at full speed to elute the DNA.

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

3.6 METHYL SPECFIC POLYMERASE CHAIN REACTION (MSP)

MSP is a novel, sensitive and inexpensive way for detection of hypermethylation in CpG islands of DNA (Herman *et al.*, 1996). This approach allows the determination of methylation patterns from very small samples of DNA. The principle of this PCR method is the is a bisulfite conversion of unmethylated cytosines into uracil in the CpG islands of DNA and then the amplification of the bisulfite treated 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 it can easily be determined whether amplification is by hypermethylated or non-methylted primers, thus determine whether our CpG's were hypermethylated or non methylated.

As described earlier PCR was carried out using methyl specific PCR (MSP) method. The modified DNA is taken into two PCR vials in equal quantity and same amount of all reagents (Table 2) is added to both the vials except in one vial methylated primers and in other non-methylated primers are used (Table 3).

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 45 sec.), annealing (59^{0}C for 45 sec.) and extension (72^{o}C for 45 sec.) and by final extension step at 72^{o}C for 4 min (Table 4).

Controls without DNA were performed for each set of PCRs along with both positive and negative controls. Each PCR reaction (10 µl) was directly loaded onto 4 % agarose gel, stained with ethidium bromide, and visualized under UV illumination.

Table 2: 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 3: Primer pairs used for amplification of the DNA Samples (Esteller *et al.,* 1999 a).

Nature of Sequence		Primer sequence
UNMETHYLATED PRIMER	Forward primer	5'-TTTGTGTTTTGATGTTTGTAGGTTTTTGT- 3'
	Reverse primer	5'-AACTCCACACTCTTCCAAAAACAAAACA- 3'
METHYLATED PRIMER	Forward primer	5'-TTTCGACGTTCGTAGGTTTTCGC- 3'
	Reverse primer	5'-GCACTCTTCCGAAAACGAAACG- 3'

Table 4: Thermal cycling conditions for Methyl Specific Poplmerase Chain Reaction (MSP).

Steps	**Temperature^{0}C**	**Time**	**Number of cycles**
1. Hot-Start	95	5 min	1
2. Denaturation	95	45 sec	35
3. Annealing	59	45 sec	
4. Extension	72	45 sec	
5. Final extension	72	4 min	1

Universal Methylated Human DNA Standard and Control with primers (**ZYMO RESEARCH**) were used as positive control and normal lymphocyte DNA as negative control.

The amplified DNA are of different base pairs in length the methylated band is 81 bp and the unmethylatd band is 93 bp and this difference in the size of the amplified DNA allowed them to be visually differentiated in presence of a 20- 1000 bp DNA ladder run parallel to the amplified PCR products on 4% ethidium bormide pre-loaded agarose gel

3.7 STATISTICAL ANALYSIS

The χ^2-test with Odds ratio was used to examine the differences in the distribution of genotypes 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.

4.1. General characteristics of Study Population

In the present study, 50 colorectal tissue samples were taken from colorectal cancer patients of Kashmir valley and 20 histopathologically confirmed normal tissues samples taken as controls General characteristics of the colorectal cancer patients were recorded in the detailed case history. In the study the case vs. control effect and gender effect of promoter hypermethylation of *MGMT* gene was studied. The data obtained is depicted in the table 5.

4.2 ANALYSIS OF *MGMT* GENE PROMOTER HYPERMETHYLATION

Analysis of the *MGMT* gene promoter hypermethylation was conducted using Methyl specific PCR (MSP) reaction. The amplicons were analysed on 4% agarose gel. Amplification was carried out using PCR-Hot Start method; the method involves heating the PCR mix without Taq DNA Polymerase upto 95^{o}C for 5 min. and then adding Taq DNA Polymerase to it. This decreases the non specific amplifications. The amplicon of methylated and non methylated primers is of different sizes i.e. 81 and 93 bp respectively and is depicted in table 6.

Figures 8 and 10 represent the gel images of cases and controls respectively.

4.2.1 General overview

Out of total of total 70 specimens studied of which 50 were cases and 20 controls. The information generated is depicted in figure 13.

To further analyse the data and to study whether gender has any role in promoter hypermethylation of MGMT gene, we split the cases and controls into two groups i.e. males and females and accordingly interpreted the promoter hypermethylation and non hypermethylation in these groups which is depicted in figure 14-18.

Table 5:-Gender study

Characteristics		Cases (50)	Controls (20)
Gender	Male	29	10
	Female	21	10

Table 6: Different lengths of fragments obtained in PCR

Nature of Sequence		Primer sequence	Size of Amplicon
UNMETHYLATED PRIMER	Forward primer	5'-TTTGTGTTTTGATGTTTGTAGGTTTTTGT- 3'	93 bp
	Reverse primer	5'-AACTCCACACTCTTCCAAAAACAAAACA- 3'	
METHYLATED PRIMER	Forward primer	5'-TTTCGACGTTCGTAGGTTTTCGC-3'	81 bp
	Reverse primer	5'-GCACTCTTCCGAAAACGAAACG-3'	

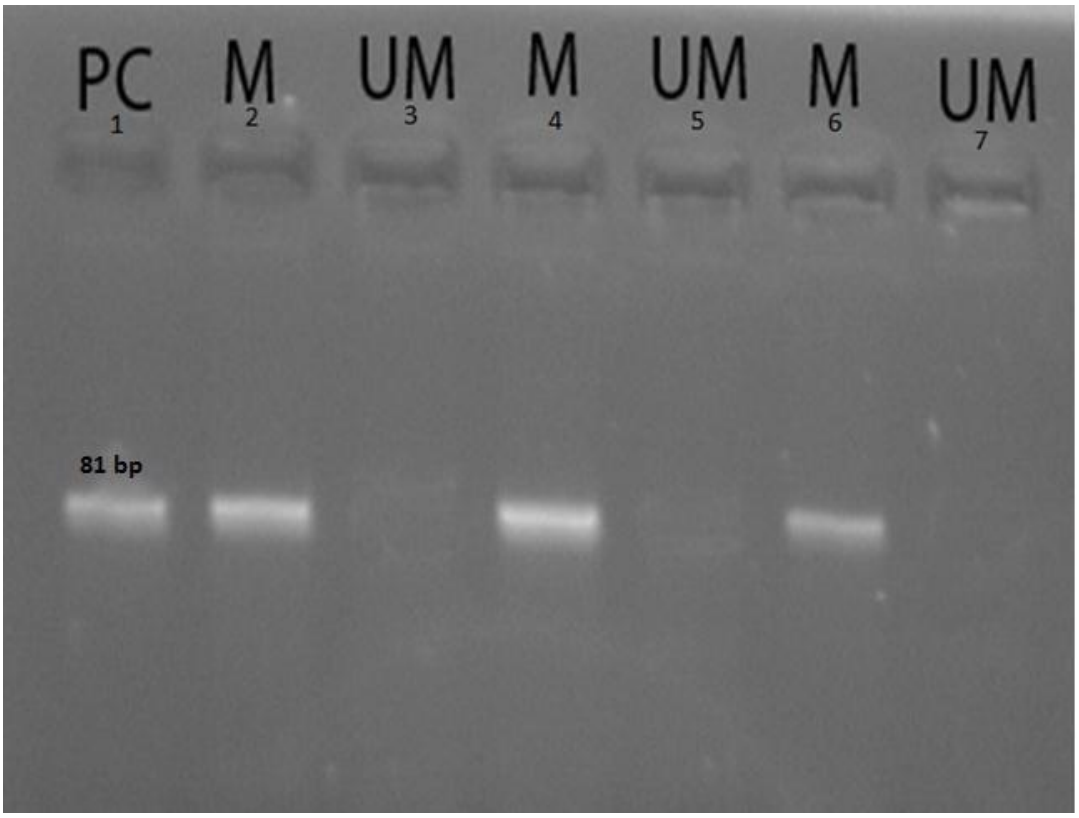

Figure 8: The cancer DNA samples amplified by MSP on 4% Agarose Gel stained with Ethidium Bromide.

Lane1		Positive control (Universal methylated human DNA) 81bp	
Lane2	CRC 5	Amplification by methylated primer	CRC sample promoter is hypermethylated
Lane3		Amplification by unmethylated primer	
Lane4	CRC 25	Amplification by methylated primer	CRC sample promoter is hypermethylated
Lane5		Amplification by unmethylated primer	
Lane6	CRC 45	Amplification by methylated primer	CRC sample promoter is hypermethylated
Lane7		Amplification by unmethylated primer	

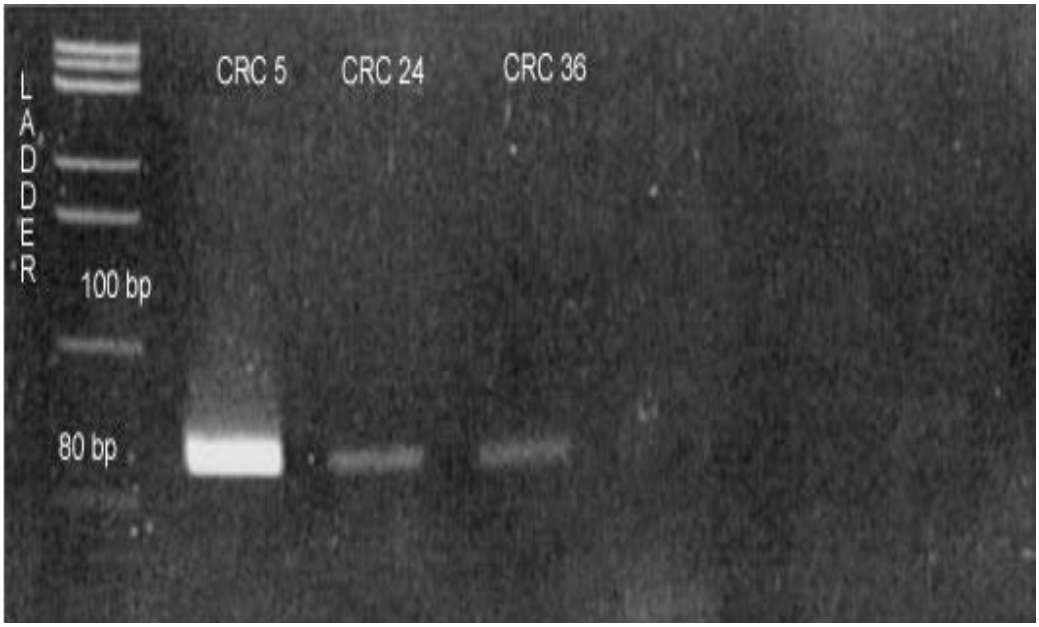

Figure 9: The cancer DNA samples amplified by MSP run along with Molecular Size DNA Ladder on 4% Agarose Gel stained with Ethidium Bromide.

lane 1	Molecular Size Marker
Lane 2	MSP Amplicon of Sample CRC 5
Lane 3	MSP Amplicon of Sample CRC 24
Lane 4	MSP Amplicon of Sample CRC 36

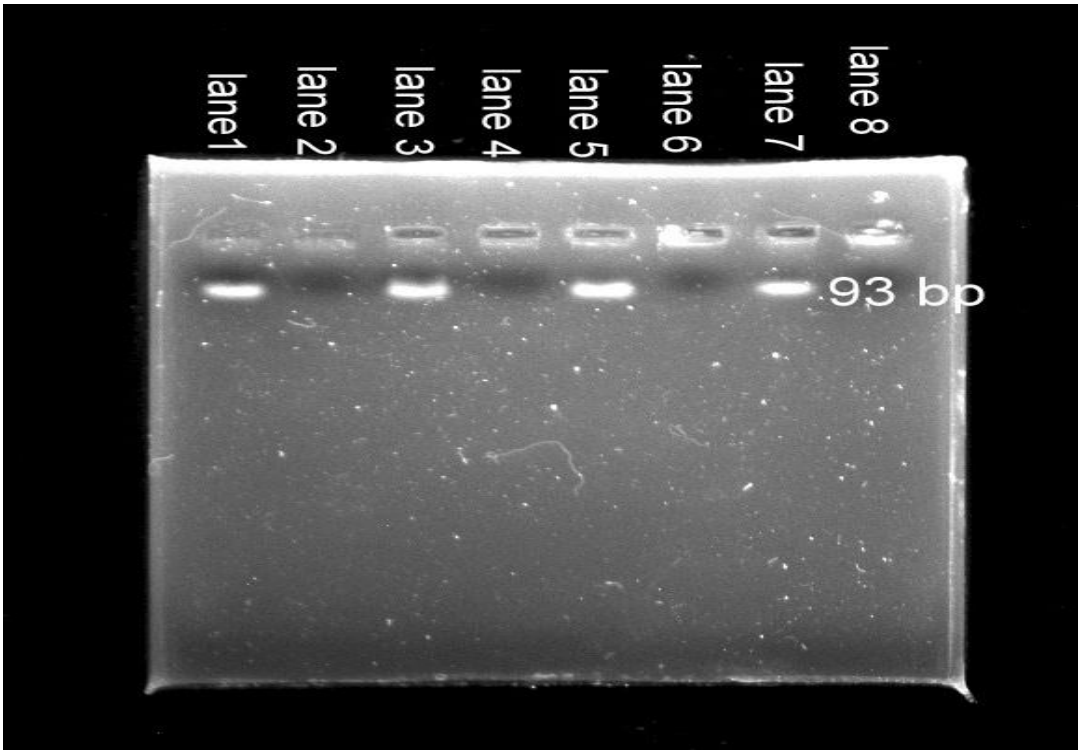

Figure 10: The Normal DNA samples amplified by MSP on 4% Agarose Gel stained with Ethidium Bromide.

Lane 1	Normal 1	Amplification by unmethylated primer	Normal sample promoter is not hypermethylated
Lane 2		No Amplification by methylated primer	
Lane 3	Normal 6	Amplification by unmethylated primer	Normal sample promoter is not hypermethylated
Lane 4		No Amplification by methylated primer	
Lane 5	Normal 15	Amplification by unmethylated primer	Normal sample promoter is not hypermethylated
Lane 6		No Amplification by methylated primer	
Lane 7	NEGATIVE CONTROL	Amplification by unmethylated primer	NEGATIVE CONTROL not hypermethylated
Lane 8		No Amplification by methylated primer	

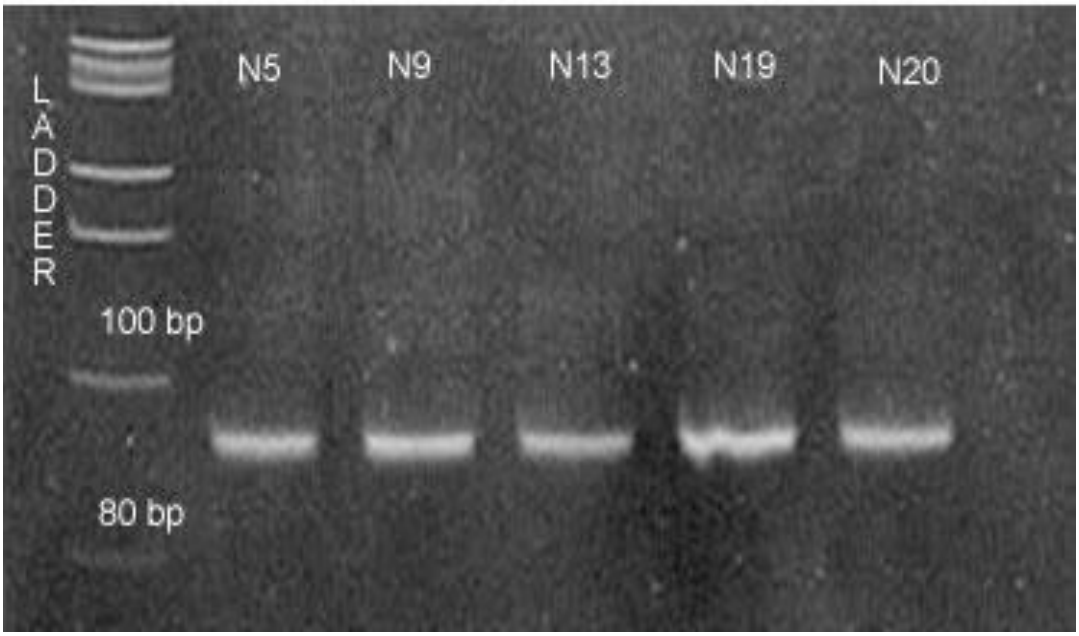

Figure 11: The Normal DNA samples amplified by MSP run along with Molecular Size DNA Ladder on 4% Agarose Gel stained with Ethidium Bromide.

lane 1	Molecular Size Marker
Lane 2	MSP Amplicon of Sample Normal 5
Lane 3	MSP Amplicon of Sample Normal 9
Lane 4	MSP Amplicon of Sample Normal 13
Lane 5	MSP Amplicon of Sample Normal 19
Lane 6	MSP Amplicon of Sample Normal 20

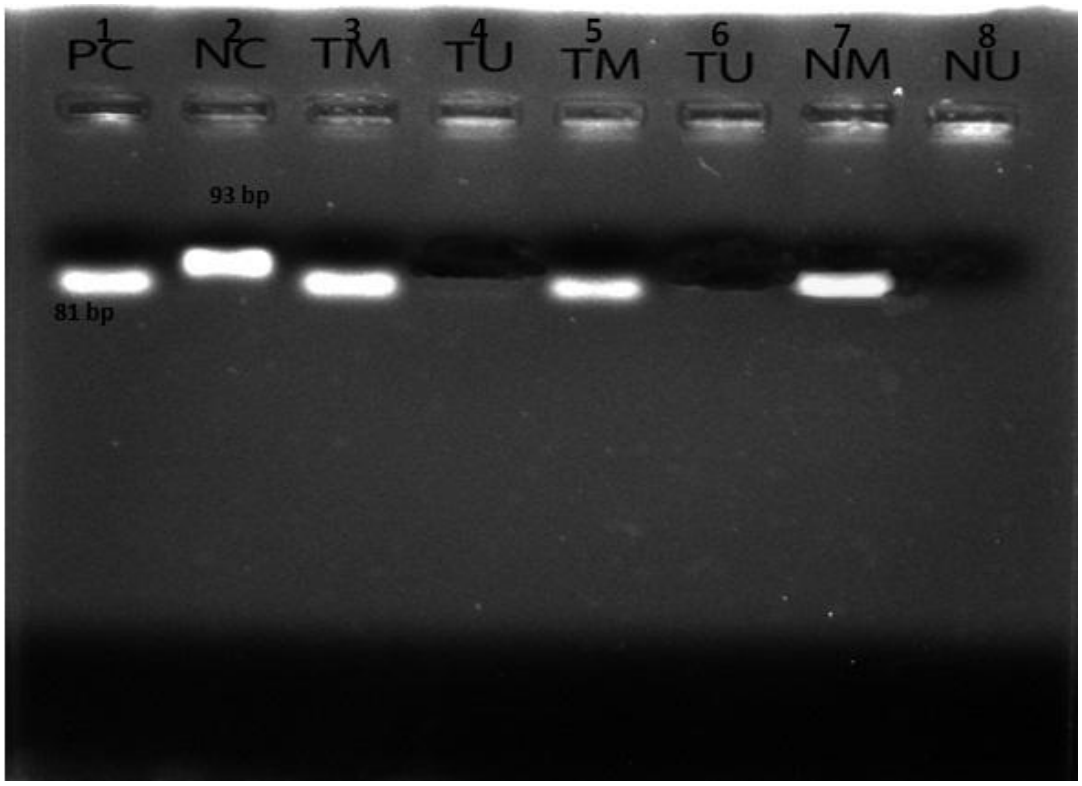

Figure 12: The Normal and cancer DNA samples amplified by MSP 4% Agarose Gel stained with Ethidium Bromide.

Lane 1		Positive control 81bp	
Lane 2		Negative control 93bp	
Lane 3	CRC 9	Amplification by methylated primer	CRC sample promoter is hypermethylated
Lane 4		Amplification by unmethylated primer	
Lane 5	CRC 19	Amplification by methylated primer	CRC sample promoter is hypermethylated
Lane 6		Amplification by unmethylated primer	
Lane 7	Normal 5	Amplification by methylated primer	NORMAL sample promoter is hypermethylated
Lane 8		Amplification by unmethylated primer	

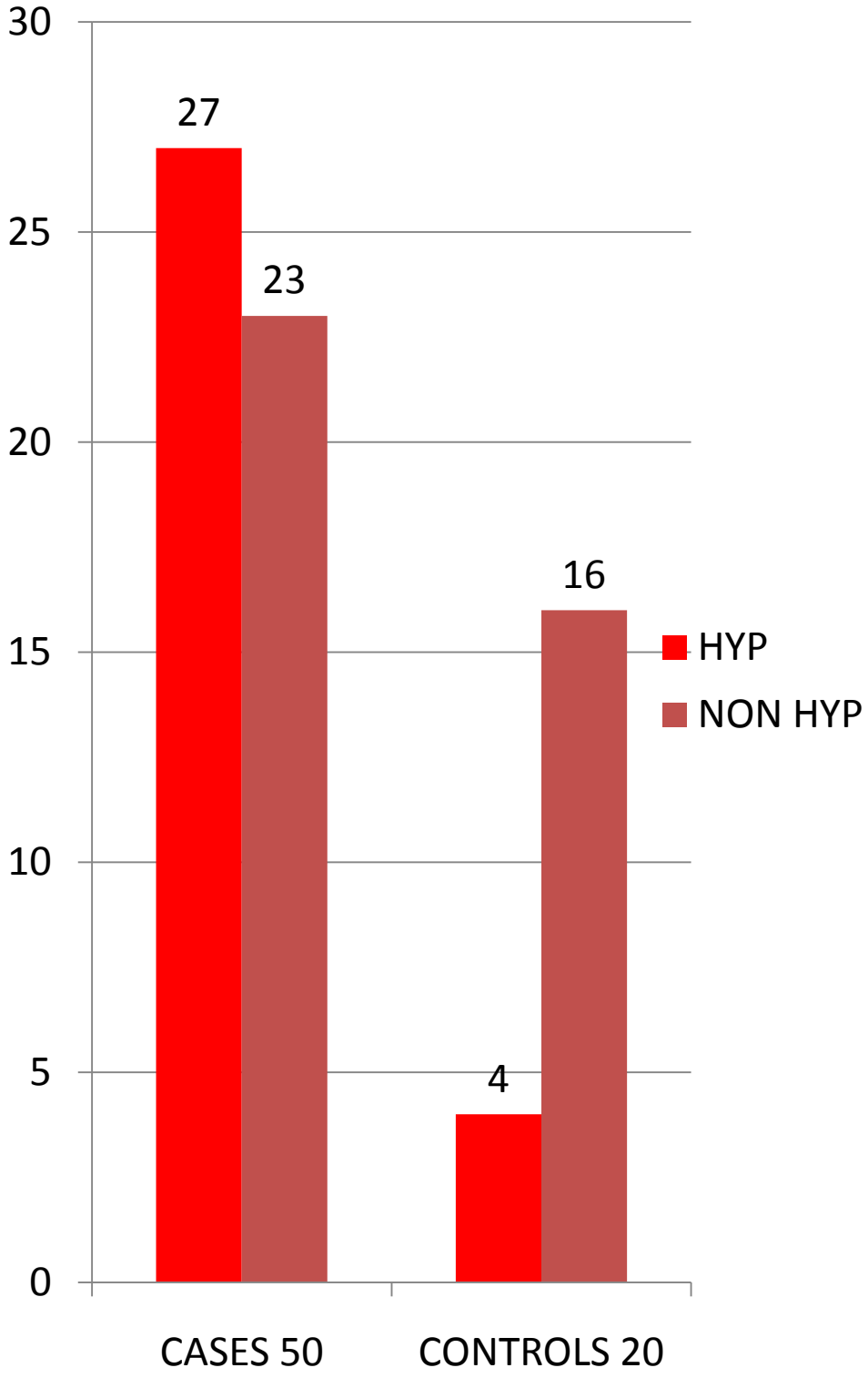

Figure 13: Bar diagram showing Case Vs Control data w.r.t promoter hypermethylation status of *MGMT* gene

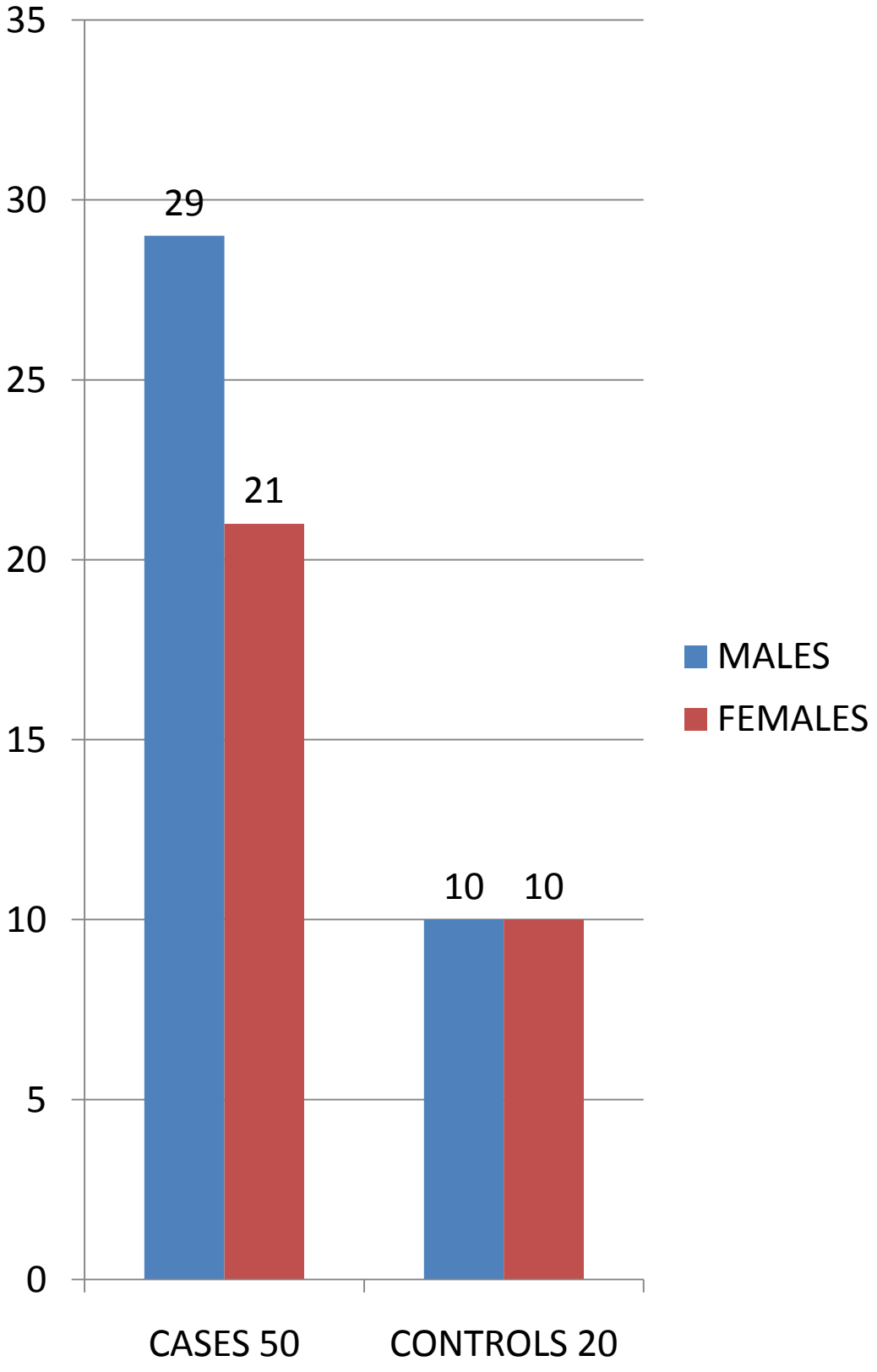

Figure 14: Bar diagram showing Male Vs Female data

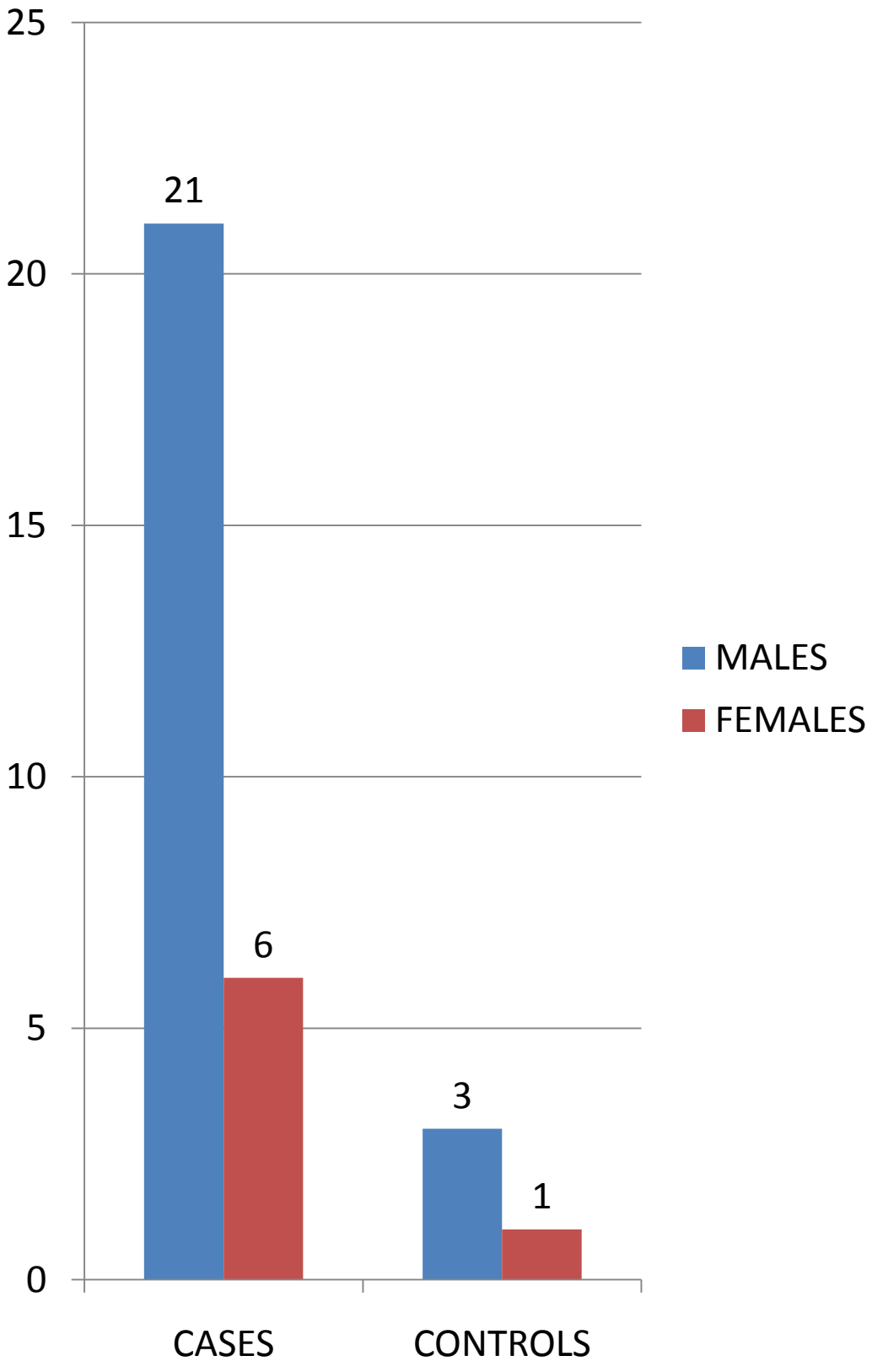

Figure 15: Bar diagram showing Hypermethylation status of promoter of *MGMT* gene of Cases Vs Control

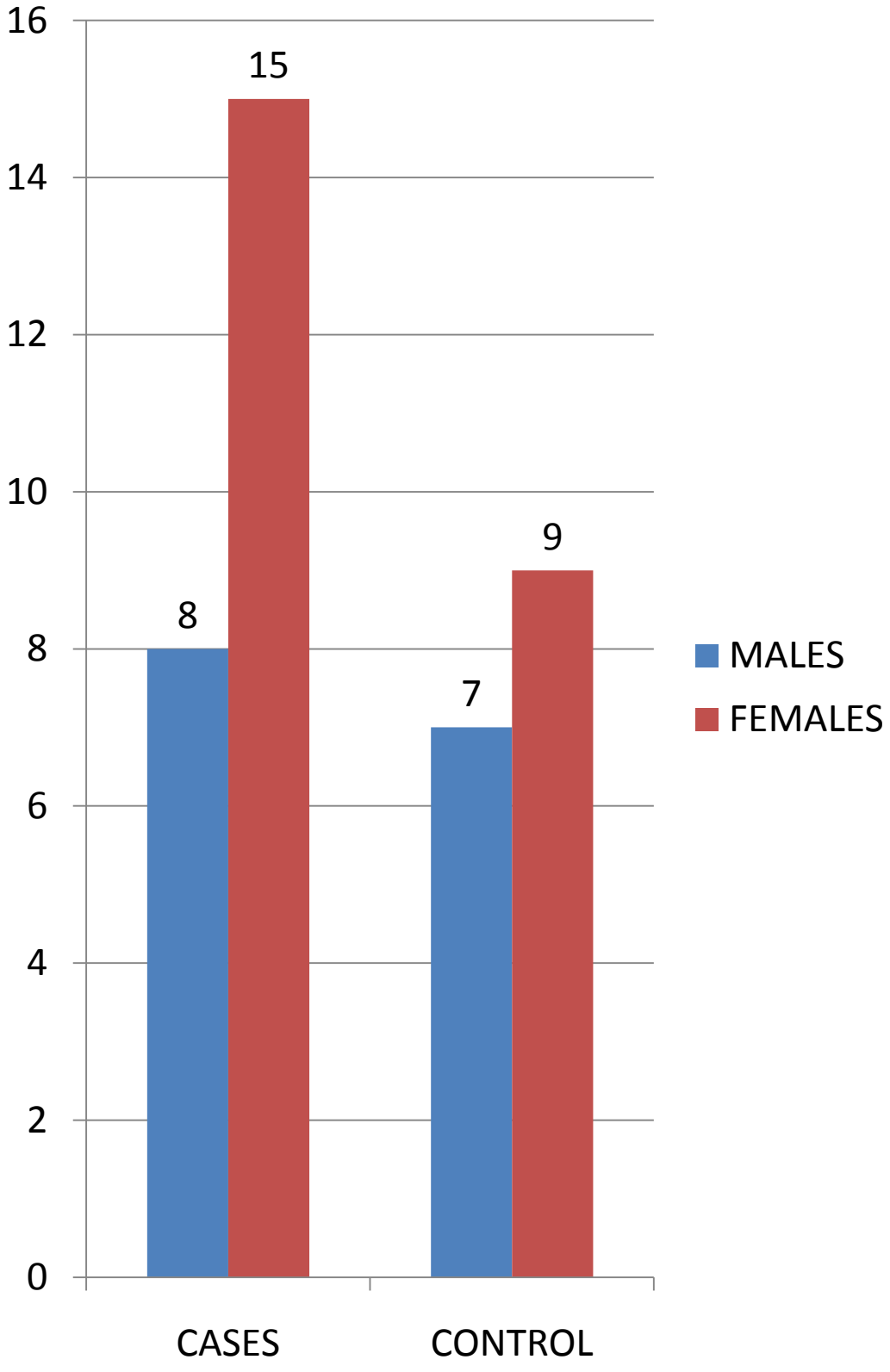

Figure 16: Bar diagram showing Non- Hypermethylation status of promoter of *MGMT* gene Cases Vs Control

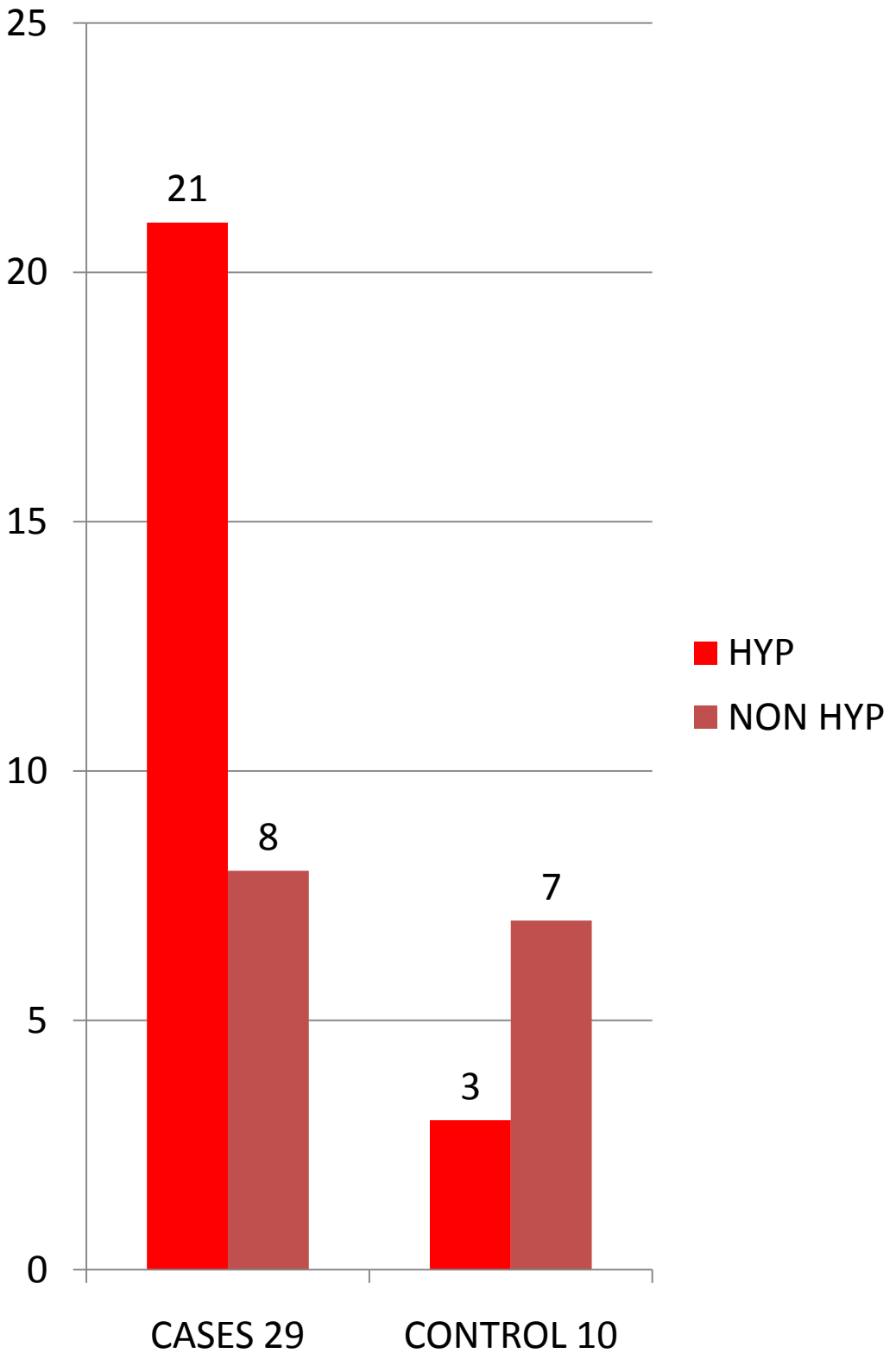

Figure 17: Bar diagram showing hypermethylation Vs non hypermethylation in both Case Vs Control data for Males

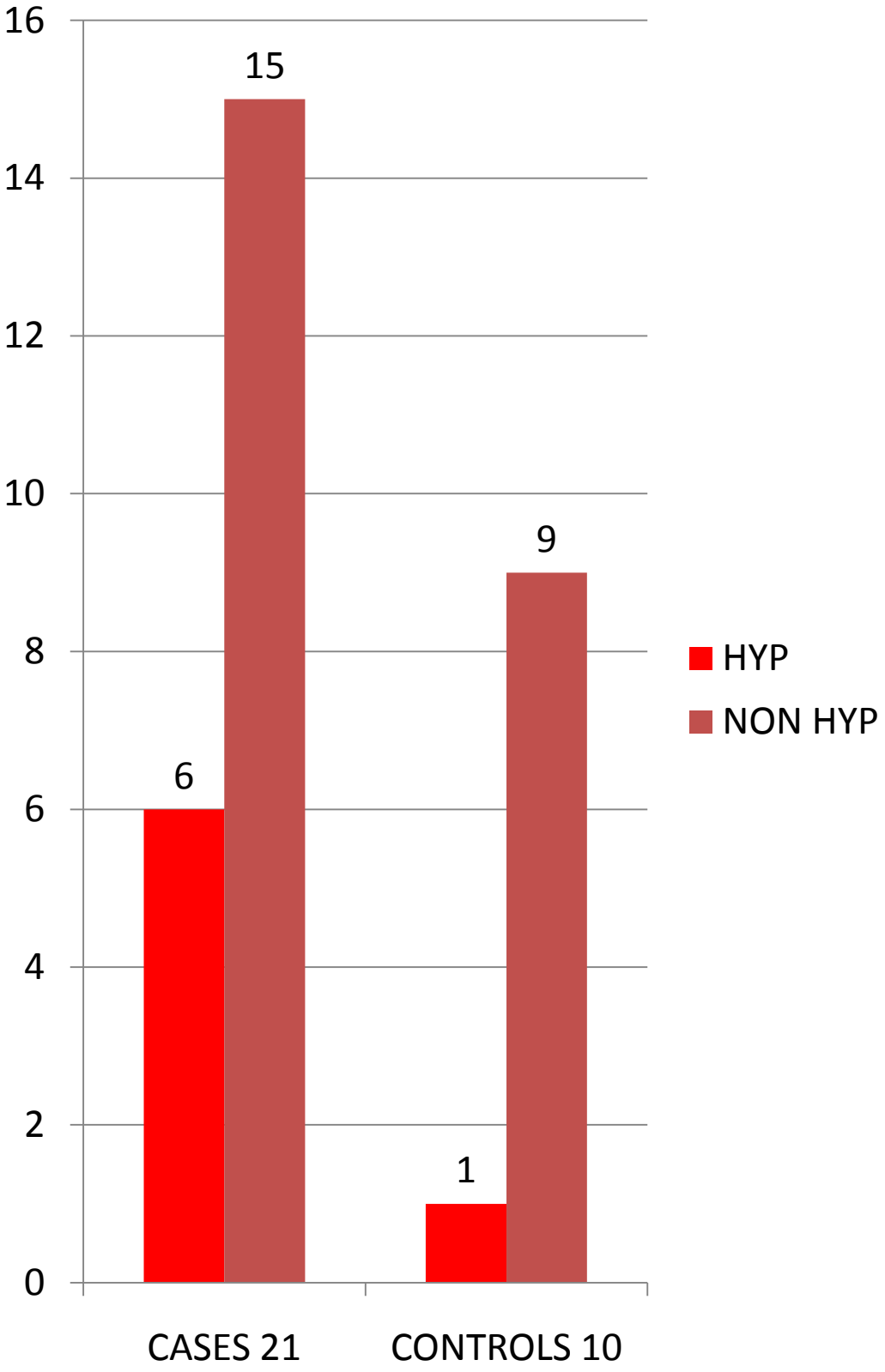

Figure 18: Bar diagram showing hypermethylation Vs non hypermethylation in both Cases and Control data in Females

4.2.1.1. Cases

In the present study 50 histopathologically confirmed cancer cases belonging to Kashmir division were analysed for promoter hypermetylation of *MGMT* gene using Methyl Specific PCR. The distribution of the samples as per their sex and status of promoter hypermethylation of the *MGMT* gene has been depicted in table 7,8 and 9. In determining the promoter hypermethylation status of the cases it was observed that out of 50 cases 27 (54%) had promoter hypermethylation of *MGMT* gene and 23 cases (46%) did not have promoter hypermethylation of *MGMT* gene(Table 7). Out of 50 cases 29 were males and 21 were females which correspond to 58% males and 42% females respectively. The number of samples having promoter hypermethylation in the *MGMT* gene in males came out to be 21 out of the 29 case samples, which corresponds to 72.41% promoter hypermethylation (Table 8) and similarly in females the numbers of cases having promoter hypermethylation of *MGMT* gene came out to be 6 out of 21 cases which correspond to 28.57% promoter hypermethylation (Table 9).

4.2.1.2. Controls

Twenty histopathologically normal controls were taken from Department of Surgery, including biopsies from Endoscopic Section and were screened for the promoter hypermethylation in the *MGMT* gene. Out of twenty controls ten were males and ten were females. Both were taken in equal numbers which corresponds to 50 % respectively. In determining the promoter hypermethylation status of the controls it was observed that out of 20 control 4 (20%) had promoter hypermethylation of *MGMT* gene and 16 cases (80%) did not have promoter hypermethylation of *MGMT* gene (Table 10). Out of 10 male controls 3 were having promoter hypermethylation in the *MGMT* gene which correspond to 30% promoter hypermethylation (Table 11) and out of 10 female control specimen just 1 came out to be positive for promoter hypermethylation in the *MGMT* gene which correspond to just 10% promoter hypermethylation (Table 12).

Table 7: Promoter Hypermethylation Vs Non Hypermethylation in Cases.

PARAMETER	NUMBER	
HYPERMETHYLATION	27 (54 %)	50 CASES
NON-HYPERMETHYLATION	23 (46 %)	

Table 8: Promoter Hypermethylation Vs Non Hypermethylation in Male Cases.

MALES (29) {CASES 50}		
PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATION	21	72.41 % (21/29)
NON-HYPERMETHYLATED	8	27.58 % (8/29)

Table 9: Promoter Hypermethylation Vs Non Hypermethylation in Female Cases.

FEMALES (21) {CASES 50}		
PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATION	6	28.57 % (6/21)
NON HYOERMETHYLATED	15	71.14 % (15/21)

Table 10: Promoter Hypermethylation Vs Non Hypermethylation in Controls.

PARAMETER	NUMBER	
HYPERMETHYLATION	4 (20%)	20 CONTROLS
NON-HYPERMETHYLATION	16 (80%)	

Table 11: Promoter Hypermethylation Vs Non Hypermethylation in Male Controls.

MALES (10) {CONTROLS (20)}		
PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATION	3	30 % (3/10)
NON HYOERMETHYLATED	7	70 % (7/10)

Table 12: Promoter Hypermethylation Vs Non Hypermethylation in Female Controls.

FEMALES (10) {CONTROLS (20)}		
PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATION	1	10 % (1/10)
NON HYOERMETHYLATED	9	90 % (9/10)

4.3. DESCRIPTIVE EPIDEMIOLOGY

As several studies have revealed that colorectal carcinogenesis is the result of the contribution of various factors such as environmental, dietary and genetic and since there is a high incidence of gastrointestinal malignancies in Kashmir, the current study was aimed at understanding the relationship if any between high incidence of colorectal cancer and the promoter hypermethylation of *MGMT* gene in Kashmiri Population.

On analyzing the data it comes out that out of 50 colorectal cancer patients registered, 29 were males and 21 were females hence the ratio comes out to be 1.38. Of all the ten districts highest number of colorectal cases turned out from Srinagar and Budgam districts of Kashmir valley which are the main urbanized towns of the valley hence indicating the role of urban lifestyle in the development of this disease. Besides this study also reveals that male gender has greater percentage of *MGMT* promoter hypermethylation as compared to females in Kashmiri population.

Colorectal cancer (CRC), commonly known as bowel cancer 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, an estimated 148,810 new cases will be diagnosed, and 49,960 deaths from colorectal cancer will occur (ACS, 2008). Most colorectal cancers (CRC) develop through multiple mutations in the normal colonic mucosa, and evolve through the adenoma-carcinoma sequence (Kinzler and Vogelstein, 1996; Fearon and Vogelstein, 1990). 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 a light on the different alternative pathways involved in the colorectal carcinogenesis and more importantly cross talk among these pathways (Risques *et al.,* 2003; 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; Wei *et al.,* 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 detox reactions are going on in our body at all the times and sometimes these lead to the production of certain harmful chemical compounds eg. 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 (Kazoni *et al.,* 2000). On the other hand we eat a variety of compounds both natural and man made and these contain a cocktail of chemicals which have a potential of acting as carcinogens like mono sodium glutamate, poly aromatic hydrocarbons, nitrosoamines (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.

The current study was aimed at understanding

- The promoter hypermethylationstatuses of MGMT gene of colorectal subjects of Kashmiri origin subjects and their corelation histopathologically confirmed controls.
- Further, analysis of role of sex if any in the promoter hypermethylation of MGMT gene.

The male to female ratio of the cancer in study was 1.38. Highest number of colorectal cases turned out to be from Srinagar and Budgam districts which are the main urbanized towns of the Valley implicating a role of urban lifestyle in etiology of this cancer, which can be further evaluated in a detailed study. The epi-genetic analysis of the cases and controls revealed that unlike other high risk regions, Kashmiri population has a different hypermethylation profile of MGMT promoter. Analysed data showed was found that 54% (27/50) of the cases had MGMT promoter hypermethylation as compared to 20% (4/20) in controls and was statistically significant with a three-fold increase in risk of developing colorectal cancer (O.R = 4.69, 95% C.I = 1.37 – 16.05, P = 0.015) by using $\chi 2$ test with Odds Ratio. This clearly indicates that promoter hypermethylation may have a link with etiology of colorectal cancer in this particular ethnic and geographical location. This can be taken into account in establishing a DNA hypermethylome as a marker for colorectal cancer if feasible.

Besides this, study was carried to analyze the effect of gender biasing w.r.t the promoter hypermethylation of *MGMT* gene. This study revealed that the male gender has generally higher incidence of MGMT promoter hypermethylation (72.41% vs. 30%) as compared to controls, with a statistically significant results and even more than three-fold increase in risk of developing this particular type of cancer (O.R = 6.13, 95% C.I = 1.26 – 29.71, P = 0.026) by using Fisher's Exact Test. As in case of the females, it was found that though percentage of promoter hypermethylation of MGMT gene of female cases was more as compared to female controls (28.57% vs. 10%), the data was not statistically significant (O.R = 3.6, 95% C.I = 0.37 – 34.93, P = 0.37) using Fisher's Exact test. Similarly when data was

analyzed for the male verses female cases of promoter hypermethylation of MGMT gene the results were statistically significant (O.R = 6.563, 95% C.I = 1.882 - 22.82, P = 0.0037) using Fisher's Exact Test.

In summary, this is the first observational study to examine the status of promoter hypermethylation of MGMT gene in colorectal cancer patients of Kashmir valley. The study revealed that urban life style may have a role in the development of this particular type of cancer as majority of the cases were from the main urban cities of the Kashmir valley i.e. Srinagar and Budgam. Data indicates that MGMT aberrant methylation may play an important role in colorectal cancer development. It also clearly demonstrates that hypermethylation of MGMT gene can be designated as epigenetic biomarker for the early diagnosis and better prognosis of colorectal cancer. The study also reveals that males have higher incidence of colorectal cancer as compared to females and also that the promoter hypermethyltion of MGMT gene is more frequent in male colorectal patients as compared to the females.

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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
Ethidium bromide	SRL
Ethyl acetate	MERCK
Ethylene diamine tetra acetate (EDTA)	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	QUALIGENS
Potassium bicarbonate	QUALIGENS
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	HIMEDIA
Sodium hydrogen carbonate	LOBA- CHEMIE
Sodium phosphate dibasic	LOBA- CHEMIE
Sodiun thiosulfate	LOBA CHEMIE
Sucrose	QUALIGENS
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

20- 1000bp DNA ladder	SIGMA ALDRICH (D7807)

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 isolated by kit based method. The kit used was **Quick- g DNA™ MiniPrep** supplied by ZYMO RESEARCH. Kit protocol was followed for DNA isolation.

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^{TM} Kit** supplied by ZYMO RESEARCH. Kit protocol was followed for bisulfite modification of isolated DNA.

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS:

Agrose 1 % / 2%:

Agarose	0.5g / 1.0g
Buffer	50ml
Ethidium bromide	10µl

Agarose was dissolved in a buffer and heated till a clear solution is formed. Ethidium bromide 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.

Ethidium Bromide

Ethidium bromide	10mg

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

50-X TAE (pH 8.0) STOCK SOLUTION:

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

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

1-X TAE (pH 8.0) WORKING SOLUTION:

50-X TAE	20ml

Final volume was made 1000ml with distilled water.

Reagents for PCR:

Stock

Deoxyribose Nucleotide Triphosphate (dNTP) 100mM each dATP, dGTP, dCTP and dTTP.

Taq polymerase (5U/µl)

10X Taq buffer (16 mmol/L Ammonium sulphate; 67 mM/L Tris- HCL, pH 8.8; 10 mM/L 2-Mercaptoethanol); 6.7 mM/ L $MgCl_2$)

Primers: 100pM in sterile deionised water (Genescript)

20- 1000bp DNA ladder (0.5µg/µl)

Department of Biochemistry

University of Kashmir

Hazratbal, Srinagar-190006

CERTIFICATE

Certified that the work in the dissertation entitled **"Elucidation of Etiology of colorectal cancer: A study on silencing of *MGMT* gene by promoter Hypermethylation "** has been carried out by Mr.. Hyder Khan under the joint supervision of Dr. Sabhiya Majid (H.O.D Department of Biochemistry, G.M.C. Srinagar), Dr. Ruby Reshi (H.O.D Department of Pathology, G.M.C. Srinagar) and Dr. Rabia Hamid (Department of Biochemistry), and the work is suitable for the award of M.Phil degree in Biochemistry.

Co-Supervisor
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DECLARATION

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

Place: SRINAGAR **HYDER KHAN**

Date:

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THANK YOU

DATE:

HYDER KHAN

TABLE OF CONTENTS

µg	Microgram
µl	Microlitre
µM	Micromolar
ACS	American Cancer Society
ACF	Aberrant Crypt Foci
ADC	Adenocarcinoma
bp	Base pair
BPB	Bromophenol Blue
cm	Centimetre
CRC	Colorectal Cancer
DDW	Double Distilled Water
DNA	Deoxyribose Nucleic Acid
dNTP	Deoxyribose Nucleotide Triphosphate
EDTA	Ethylene Diaminetetraacetic Acid
EtBr	Ethidium Bromide
FAP	Familial Adenomatous Polyposis
Fig.	Figure
g	Gram
HCl	Hydrochloric Acid
Hr	Hours

Kb	Kilo base pair
LCA	Lithocholic acid
Kd	Kilo Dalton
M	Molar
mg	Miligram
$MgCl_2$	Magnesium chloride
min	Minutes
ml	Mililitre
mM	milimolar
mRNA	Messenger Ribonucleic Acid
MSP	Methyl Specific Polymerase Chain Reaction
NaCl	Sodium chloride
NCI	National Cancer Institute
ng	Nanogram
O.D	Optical Density
O.R	Odds Ratio
^{o}C	Degree Celsius
PAGE	Poly Acrylamide Gel Electrophoresis
PAHs	Poly Aromatic Hydrocarbons
PCR	Polymerase Chain Reaction

pH	Potenz Hydrogen
pM	pico Mole
rpm	Revolutions per minute
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
SEER	Surveillance Epidemiology and End Report
Sec	Second
TAE	Tris Acetate EDTA
Taq	*Thermus aquaticus*
TBE	Tris-Borate-EDTA
Tm	Melting Temperature
Tris	Tris(hydroxymethylaminomethane)
WHO	World Health Organisation

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Prevalence of Promoterhypermethylation of *MGMT* Gene in Colorectal Cancer Patients of Kashmir Valley.

Colorectal cancer, one of the most aggressive cancers, occurs with a high incidence in most countries. Colorectal cancer (CRC) is one of the leading malignancies worldwide. Cancer development and progression is dictated by series of alterations in genes such as tumor suppressor genes, DNA repair genes, oncogenes and others. In this study, efforts were made to identify promoter hypermethylation of CpG islands of *MGMT* gene in CRC patients among the Kashmiri population. Methylation status of CpG islands in the promoter region of *MGMT* gene in colorectal cancers and normal corresponding colonic mucosa was analysed. Fresh tissue samples were obtained from 50 patients (age of 21 to 81 years) undergoing resective surgery for CRC with primary colorectal adenocarcinonia and corresponding histopathologically normal tissues. Methylation-specific polymerase chain reaction (MSP) was used for analysis of the promoter methylation status of *MGMT* gene. The male to female ratio of the disease came out to be 1.38. The epigenetic analysis of the cases and controls revealed that unlike other high risk regions, Kashmiri population has a different hypermethylation profile of *MGMT* gene promoter hypermethylation. The frequency of cases with *MGMT* promoter hypermethylation was more as compared to controls (54% vs. 20%) and was statistically significant (O.R = 4.69, 95% C.I = 1.37 – 16.05, P = 0.015) using χ^2 test with Odds Ratio. It was also found that the frequency of male cases with promoter hypermethylation of *MGMT* gene was more as seen against male controls (72.41% vs. 30%), which also showed statistically significant results (O.R = 6.13, 95% C.I = 1.26 – 29.71, P = 0.026) using Fisher's Exact test, though the frequency of promoter hypermethylation of *MGMT* gene of female cases was more as compared to female controls (28.57% vs. 10%), the data was found not to be statistically significant (O.R = 3.6, 95% C.I = 0.37 – 34.93, P = 0.37) using Fisher's Exact. While for the male verses female cases of promoter hypermethylation of *MGMT* gene the results were statistically significant (O.R = 6.563, 95% C.I = 1.882 - 22.82, P = 0.0037) using Fisher's Exact Test.

In this study it was concluded that male gender is generally associated with higher methylation levels for most CpG islands hypermethylation of *MGMT* gene in normal

as well as cancerous colonic mucosa. The results indicate that *MGMT* aberrant methylation may play an important role in colorectal cancer. This study clearly demonstrates that promoter hypermethylation of *MGMT* gene can be designated as epigenetic biomarker for early diagnosis and better prognosis of the disease.

Colorectal cancer (CRC), also called colon cancer or large bowel cancer includes cancerous growths in the colon, rectum and appendix. With 655,000 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 death in the Western world (WHO, 2006; NCI, 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 148, 810 new cases were diagnosed in U.S, and almost 49,960 deaths from colorectal cancer were speculated (American Cancer Society, 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 and Jan, 1990*;* Salam *et al.,* 2009*;* Mir *et al.,* 2005).Colorectal Cancer in Kashmir valley is the third most common GIT cancer after esophageal and gastric cancer as per reports (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 (Mustafa *et al.,*2007*;* Vogelstein *et al.,* 1988*;* Fearon and Vogelstein, 1990). 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. In higher order eukaryotes, DNA is methylated only at cytosines located 5' to guanosine in the CpG dinucleotide. 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 transcription inactivation of gene (Zingg and Jones., 1997). It is a complex process catalyzed by DNA methyltransferase (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 and Bird, 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).

1.2 MGMT

O^6-methylguanine-DNA methyltransferase (MGMT) is a ubiquitous DNA repair protein that removes mutagenic and cytotoxic adducts from the O^6-guanine in DNA, the preferred point of attack of many carcinogens and alkylating chemotherapeutic agents (Esteller, 2003; Esteller and Hermann, 2004). Methylation of CpG islands within the promoter region may lead to silencing of gene transcription through a complex process involving chromatin condensation and histone deacetylation (Jones *et al.,* 1998; Wade *et al.,* 1999). 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; Baylin *et al.,* 2001). Epigenetic modification can cause inactivation of DNA repair genes, such as O6-methylguanine DNA methyltransferase (MGMT) (Esteller *et al.,* 1999 a).Human MGMT gene possess a CpG island in the promoter region (Harris *et al.,* 1991) and it has been reported that the methylation of discrete regions of the MGMT CpG island is associated with the silencing of the gene in cell lines (Costello *et al.,* 1996; Qian and Brent, 1997; Watts *et al.,* 1997). Methylation of MGMT promoter region in colorectal cancer tissue has been known and documented in several independent studies (Shen *et al.,* 2005; Suehiro *et al.,* 2008). MGMT (O^6-methylguanine DNA methyltransferase) is a DNA repair enzyme and is involved in the removal of alkyl adducts from the O6 position of guanine. These adducts can be mutagenic and cytotoxic (WHO, 2006). The persistence of O^6 methyl guanine adducts, resulting from alkylating agents, may cause DNA polymerase to misread the base pairing because of the altered hydrogen-bonding properties of a base that contains an additional methyl or ethyl group. Thus, O^6-methylguanine is read as an adenine and mis-pairs with thymine (Horsfall *et al.,* 1990). Supporting this data, the most common mutations caused by alkylating agents are G:C to A:T transitions (Horsfall *et al.,* 1990), exemplified in the frequent generation of G to A transitions in the oncogene k-Ras when the carcinogen N-methyl nitrosourea (that forms O^6-methylguanine adducts) is used in experimentally induced tumor systems (Mitra *et al.,* 1989). Avoidance of the mutagenic effect is directly related to the presence of a functional MGMT protein (Pegg *et al.,* 1995). MGMT removes alkyl groups by

transferring the alkyl group from the O^6-guanine in DNA to an active cytosine within its own sequence in a reaction that inactivates one MGMT molecule for each lesion repaired (Pegg, 1990). Thus, the ability of a cell to withstand such damage is directly related to the number of MGMT molecules it contains and to the rate of de novo synthesis of MGMT. The amounts of MGMT protein differ according to cellular type (Gerson *et al.,* 1986; Citron *et al.,* 1991) and are decreased in some tumors, with respect to their normal tissue counterpart (Citron *et al.,* 1991; Citron *et al.,* 1992). In vitro assays show that endogenous MGMT expression protects mammalian cell lines from spontaneous G: C to A: T transitions in the APRT gene (Aquilina *et al.,* 1992). Animal models also show that transgenic mice over expressing MGMT are protected against O^6 methyl guanine-DNA adducts caused by methyl nitrosourea (Dumenco *et al.,* 1993) and against G to A mutations in K-ras in aberrant colorectal crypt foci and lung tumors (Zaidi *et al.,* 1995; Liu *et al.,* 1999). It has recently shown that MGMT is transcriptionally silenced by promoter hypermethylation in a wide spectrum of human neoplasms (Esteller *et al.,* 1999 a) and provided the first evidence in human primary tumors of the linkage between MGMT epigenetic inactivation and the appearance of G to A mutations in K-ras (Esteller *et al.,* 2000; Nagasaka *et al.,* 2003) found that methylated MGMT was significantly related to lower risk of recurrence in colorectal cancer.

In the case of colorectal tumors, the pro-mutagenic lesion affecting the O^6-guanine can be caused by alkylating agents provided from dietary nitrates that are reduced in the proximal colon by bacteria or by nitrosation of amines and amides derived from protein catabolism (Bartsch *et al.,* 2004*;* Rowland *et al.,* 1991*;* Ward *et al.,* 1989). So the main aim of the study was:

- To study the promoter hypermethylation of MGMT gene in colorectal patients of Kashmir that may help in early diagnosis and better prognosis of the disease so that further preventive measures could be taken.