Elucidation of etiology of gastric cancer: A study on epigenetic silencing of TCF4 gene

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

By

Rabia Farooq

UNDER THE JOINT SUPERVISION OF

Prof. Sabhiya Majid
Dr. Shajrul Amin

DEPARTMENT OF BIOCHEMISTRY
GOVERNMENT MEDICAL COLLEGE, SRINAGAR
(RESEARCH CENTRE, UNIVERSITY OF KASHMIR)

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(NAAC Accredited Grade "A")
Srinagar- 190006(J&K)
www.kashmiruniversity.ac.in
www.biochemku.org.in

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Department of Biochemistry

University of Kashmir

Hazratbal, Srinagar-190006

CERTIFICATE

Certified that the work embodied in the dissertation entitled **"Elucidation of etiology of gastric cancer: A study on epigenetic silencing of TCF4 gene"** has been carried out by **Ms. Rabia Farooq** under the joint supervision of **Prof. Sabhiya Majid and Dr. Shajrul Amin** and the work is suitable for the award of M.Phil degree in Biochemistry.

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

Prof. Sabhiya Majid
Supervisor
Professor & Head
Department of Biochemistry
Govt Medical College Srinagar

Dr. Shajrul Amin
Co-Supervisor
Sr. Assistant Professor
Department of Biochemistry
University of Kashmir

Prof. Mohammad Afzal Zargar
Head
Department of Biochemistry
University of Kashmir

Department of Biochemistry

University of Kashmir

Hazratbal, Srinagar-190006

DECLARATION

I, Rabia Farooq, declare that the work embodied in this dissertation entitled **"Elucidation of etiology of gastric cancer: A study on epigenetic silencing of TCF4 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 Department of Biochemistry, University of Kashmir, Srinagar and is original. The work embodies the results of my observations which are advancement to the previous knowledge in the subject.

Place: Srinagar **Rabia Farooq**

Date:

DEDICATED TO MY PARENTS

Who cherished my dreams through sacrifice

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.

AIDS	Acquired Immuno Deficiency Syndrome
AJCC	American Joint Committee on Cancer
bHLH	Basic Helix Loop Helix
bp	Base pair
C	Cytosine
cag A	Cytotoxin associated gene A
CDKN2A	Cyclin dependent kinase inhibitor
CHD1	Chromodomain-helicase-DNA-binding protein 1
cm	Centimetre
CREB	cAMP response element-binding protein
DNA	Deoxyribose Nucleic Acid
DNMTs	DNA methyl transferases
dNTP	Deoxyribose Nucleotide Triphosphate
EBV	Epstein Barr Virus
EDTA	Ethylene Diaminetetraacetic Acid
Fig.	Figure
g	Gram
GC	Gastric Cancer
GIT	Gastrointestinal Tract
GSTP1	Glutathione S-transferase P
µg	Microgram
µl	Microlitre
µM	Micromolar
HCl	Hydrochloric Acid
HDAC	Histone deacetylase
HDGC	Hereditary Diffuse Gastric Carcinoma

HIV	Human Immunodeficiency virus
HLH	Helix Loop Helix
HMG	High Mobility Group
HP	Helicobacter pylori
HPLC	High performance liquid chromatography
HRT	Hormone replacement therapy
IARC	International Agency for research on cancer
ITF2	Immunoglobin Transcription Factor 2
Kb	Kilo base pair
Kd	Kilo Dalton
M	Molar
MBD	Methyl Binding Domains
MeCP1	Methyl-CpG-binding protein
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minutes
ml	Millilitre
mM	milimolar
ng	Nanogram
O.D	Optical Density
O.R	Odds Ratio
^oC	Degree Celsius
PCR	Polymerase Chain Reaction
PHS	Pitt Hopkins Syndrome
Rb	Retinobalstoma
SAM	S –adenosyl methyl transferase

SDS	Sodium Dodecyl Sulphate
sec	Second
SKIMS	Sheri – Kashmir Institute of Medical Sciences Soura
SMHS	Shri Maharaja Hari Singh
SNP	Single nucleotide polymorphism
Taq	*Thermus aquaticus*
TCF	Transcription Factor
Tm	Melting Temperature
5YSR	5 year survival
TNM	Tumour node metastasis
TRD	Transcriptional repressor domain
U	Uracil
UK	United Kingdom
UV	Ultraviolet
WHO	World Health Organisation

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ABSTRACT

Gastric cancer (GC) is the second most common cancer among population, and it causes 800,000 deaths worldwide annually. Most deaths from stomach cancer occur in men of age 55 and older. The risk for developing gastric cancer appears to be affected by several factors. People with a family history of gastric cancer have an increased risk of developing this disease. Besides, *Helicobacter pylori* infection also can be the cause of this disease. Cancer development is caused by series of genetic alterations in genes like in oncogenes, tumor suppressor genes, DNA repair genes, etc. Besides epigenetic alterations are also taken into consideration. The *TCF4* gene is frequently found to be inactivated by promoter methylation in a broad range of human tumors. The *TCF4* gene belongs to a family of genes called bHLH . It is involved in the development and functioning of many different cell types. Exogenous expression of bHLH family proteins can promote cell cycle arrest and apoptosis.

The aim of this study was to identify promoter hypermethylation in CpG islands of *TCF4* gene in gastric cancer patients among the Kashmiri population. In this study methylation status of CpG islands in the *TCF4* gene in histopathologically confirmed 50 gastric cancer samples and histopathologically confirmed 30 normal gastric tissues was analyzed. Methylation Specific Polymerase (MSP) chain reaction was used for analysis of *TCF4* promoter hypermethylation status. In the current study, it was found that 66% (33/50) of the cases had *TCF4* promoter hypermethylation while as 34% (17/50) of the cases were unmethylated. The study also revealed that 20% (6/30) of the controls also had promoter hypermethylation of CpG islands of *TCF4* gene and 80% (24/30) did not show promoter hypermethylation of CpG islands of *TCF4* gene. The association of promoter hypermethylation with gastric cancer was evaluated by χ^2 (Chi square) test with Odds ratio and was found to be significant (P=0.0001, Odds ratio=17.47, 95%C.I=3.620-84.32). Among 33 male cases and 17 female cases, the association of promoter hypermethylation with gastric cancer was evaluated using Fischer's exact test and was found to be significant in both males and females. However, the occurrence of *TCF4* promoter

hypermethylation was found to be unequally distributed in males and females with more frequency in males than females but the difference was not statistically significant (p= 0.08).

From this hypermethylation study it is inferred that *TCF4* gene promoter is often methylated in gastric cancer patients and thus these results suggest that *TCF4* promoter hypermethylation may contribute to the process of carcinogenesis in gastric cancer. The results also suggest that hypermethylation of *TCF4* gene can be designated as epigenetic biomarker for the screening, diagnosis and prognosis of gastric cancer.

CHAPTER: 1

INTRODUCTION

Cancer (medical term: malignant neoplasm) is a general term for large group of diseases characterised by self sufficiency in growth signals, insensitivity to growth inhibitory (antigrowth signals),evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis and tissue invasion (Hanahan and Weinberg, 2000). Cancer affects people of all ages, and a few types of cancer are more common in children. Cancer occurs due to failure of regulation of genes which control cell growth and differentiation (Croce, 2008). The affected genes include oncogenes and tumor suppressor genes. Malignant transformation can also occur through the formation of novel oncogenes, the inappropriate over-expression of normal oncogenes, or by the under-expression of tumor suppressor genes. Thus, changes in many genes are required to transform a normal cell into a cancer cell (Knudson, 2001). Genetic changes can occur at different levels and by different mechanisms like the gain or loss of an entire chromosome during mitosis, mutations, deletion or gain of a portion of a chromosome, translocation, inversion etc. In the global incidence of cancer, esophageal cancer is the sixth, colorectal cancer is the third, and gastric cancer is the second most common tumor (Chan and Rashid, 2006). GIT cancers account for about 20% of all cancers worldwide.

Gastric cancers are typically carcinomas which arise from the epithelium, or surface lining, of the stomach. It starts from one of the common cell types found in the lining of the stomach. It has a very poor prognosis with 800,000 deaths per year (Cancer Fact sheet, 2009). Gastric cancer is asymptomatic disease and its prognosis is related to tumor extent and includes both nodal involvement and direct tumor extension beyond the gastric wall (Nakamura *et al*., 1992; Slewart *et al*., 1998).

DNA methylation is one of the most commonly occurring epigenetic event taking place in the mammalian genome. In prokaryotes DNA methylation provides a way to protect host DNA from digestion by restriction endonucleases that are designed to eliminate foreign DNA, and in eukaryotes it functions in the regulation/control of gene expression (Costella *et al*., 2001). DNA methylation occurs in promoter CpG islands which are 0.5-2 kb regulatory regions, present in the 5'- region of approximately 40% of promoters of mammalian genes (Jones and Laird, 1999; Esteller, 2002; Herman and Baylin, 2003; Fatemi *et al*., 2005). CpG dinucleotide content in CpG islands is about of at least 60%, whereas the rest of the genome has much lower CpG

frequency, a phenomenon called CG suppression (Feil ***et*** *al*., 2007). These CpG islands are targets of methylation for their proper expression. It has been demonstrated that aberrant DNA methylation is a widespread phenomenon in cancer and may be among the earliest changes to occur during oncogenesis (Stirzaker, 1997). It is now realized that CpG island hypermethylation also causes change in chromatin structure and histone modifications which includes histone H3 and H4 deacetylation (Johnstone, 2002) histone methylation (Kondo *et al*., 2003), histone H4 sumoylation (Shiio and Eisenman 2003) and reduced histone H3 lysine 4 methylation (Boggs *et al*., 2002; Liang *et al*., 2004), collectively resulting in a transcriptionally silenced state, phenomenon being termed as epigenetic silencing. Epigenetics has evolved as a rapidly developing area of research. The ability to detect and quantify DNA methylation efficiently and accurately has become essential for the study of cancer, gene expression, genetic disease, as well as many other important aspects of biology. To date a number of methods have been developed to detect/quantify DNA methylation including HPLC (Fraga *et al*., 2000) and methylation sensitive arbitrarily primed PCR (Gonzalgo *et al*., 1997). However the most common technique used today remains the bisulfite conversion method (Frommer, 1992). This method is ideal for mapping the normal and aberrant patterns of methylation. This technique involves treating methylated DNA with bisulfite which converts unmethylated cytosines into uracil without causing any change in methylated cytosines. Once converted the methylation profile of the DNA can be determined by PCR amplification followed by DNA sequencing.

The ubiquity of DNA methylation changes has opened the way to a host of innovative diagnostic and therapeutic strategies. Aberrant DNA methylation patterns provide three powerful diagnostic applications as classification markers, as sensitive detection markers, and risk assessment markers as the field moves to human epigenome projects. Besides acting as diagnostic markers, epigenetic changes in cancer cells also provide novel targets for drug therapy (Stirzaker *et al*., 1997; Gerasimova *et al*., 2001; Di Croce *et al*., 2002; Gonzalez *et al*., 2003). Recent advances in research makes DNA methylation markers as powerful future tools in the clinic as the field of cancer epigenetics is evolving rapidly on several fronts (Jones and Baylin, 2002; Fruhwald, 2003; Laird, 2005). Its wide applicability and potential importance will possibly lead to increasing clinical impact in the near future (Hanash, 2004). Thereby, advances in our

understanding of chromatin structure, histone modification, transcriptional activity and DNA methylation have resulted in an increasingly integrated view of epigenetics.

Epigenetic silencing through DNA methylation can begin very early in tumor progression and may affect multiple genes involved in different cellular pathways including cell cycle control, DNA repair and many others (Baylin *et al*., 1998; 2001). Promoter hypermethylation can cause transcriptional inactivation/silencing of various cell cycle control genes, tumor suppressor genes like *RASSF1A, p16, hMLH1, CDH1* etc. Many genes are modified in stomach cancer and one of such gene is *TCF4*. *TCF4* is a protein coding gene which codes for protein Tcf4 that binds to specific regions of DNA and helps to control the activity of many other genes, which helps in cell differentiation, DNA dependent transcription, initiation etc. On the basis of this action, the *TCF4* protein is known as a transcription factor. *TCF4* is a downstream target of the Wnt/β-catenin pathway and is found to be deregulated in human colon cancers (Kolligs, 2002). Interaction of nuclear β-catenin and *TCF4* is believed to trigger the transcription of multiple cancer associated genes, including CD44, cyclin D, c myc. *TCF4* null mice show developmental defects of small intestine (Korinek *et al*., 1998).

TCF4 gene is located on chromosome 18q21.2 and spans 437 kbp. Although the expression of *TCF4* is ubiquitous, its levels vary considerably between tissues. Epigenetic mechanisms of gene activation, including promoter hypermethylation, are undoubtedly important in cancer development and represent an alternative means of inactivating important genes. However, before accepting the conclusion that promoter hypermethylation is invariably the cause of gene inactivation, it is worth evaluating the data a bit more critically. Nevertheless, the standard of proof for establishing that hypermethylation of promoter of any given gene has a critical role in loss of gene expression and cancer development should probably be set quite high, regardless of whether the gene is a cell cycle regulatory gene or tumor suppressor gene.

The present study is an attempt to analyze the *TCF4* gene promoter hypermethylation in gastric carcinoma patients of Kashmir valley, so that it can be used as epigenetic marker for the screening of gastric cancer. This may help in prognosis and diagnosis of the disease so that further preventive measures could be taken.

CHAPTER:2

REVIEW OF LITERATURE

2.1 Gastric Cancer

Advances in diagnostic and treatment technologies have resulted in excellent long term survival for gastric cancer but it is still the second most cause of cancer death in the world (Tominaga, 1998). Gastric cancers are typically carcinomas which arise from the epithelium of the stomach. About 95% of stomach cancers are of adenocarcinoma type (Si-Chun *et al.*, 1965), which starts from one of the common cell types found in the lining of the stomach. It is a common cancer of the digestive tract worldwide and is common in Japan (Eurogast study group, 1993), Chile, and Iceland, although it is uncommon in the United States. Several different types of cancer can occur in the stomach. There are number of rarer types of cancer that can affect the stomach. These include:

- Soft tissue sarcomas, of which the commonest are leiomyosarcomas
- Gastrointestinal stromal tumours .
- Lymphomas such as mucosa associated lymphoid tissue lymphomas (4%)
- Carcinoid tumors (3%) (Kumar, 1994)

Gastric cancer has a very poor prognosis. It is more prevalent in males than females (Jayaramam *et al*., 2005) mostly over age of 50 years. Stomach cancer tend to develop slowly over many years. Before a true cancer develops pre-cancerous changes often occur in the lining of the stomach. These early changes rarely cause symptoms and often undergoes undetected, so its prognosis is poor. The overall five-year survival rate (5YSR) is approximately 30%, with most patients dying within the first year of diagnosis (Macdonald *et al*., 2004).

Cancers as a group account for approximately 13% of all deaths each year with the most common being: lung cancer (1.3 million deaths), stomach cancer (803,000 deaths), colorectal cancer (639,000 deaths), liver cancer (610,000 deaths),and breast cancer (519,000 deaths) (WHO, 2006). Over half of cases occur in the developing world (Devesa and Silverman, 1978; Jemal *et al*., 2011). Cancer is regarded as a disease that must be "fought" to end. The estimates of cancer cases for all sites for Indian males are 462,408; 497,081 and 534,353 for the years 2010, 2015 and 2020, respectively. The corresponding estimates of cancer cases for females are 517,378; 563,808 and 614,404. Further, the total cancer cases are likely to go up from 979,786

cases in the year 2010 to 1,148,757 cases in the year 2020 (Ramnath Takiar *et al*., 2010)

2.1.1 Symptoms

- Abdominal fullness or pain
- Dark stools
- Difficulty swallowing, especially if it increases over time
- Excessive belching
- General decline in health
- Loss of appetite
- Nausea and vomiting
- Premature abdominal fullness after meals
- Vomiting blood
- Weakness or fatigue
- Weight loss (unintentional)

2.1.2 Types of Gastric cancer

This malignancy exists in two principal forms (Lauren, 1965):

- Type I (intestinal)
- Type II (diffuse)

2.1.2.1 Intestinal, expansive, epidemic-type gastric cancer

Is associated with chronic atrophic gastritis, retained glandular structure, little invasiveness, and a sharp margin and is associated with most environmental risk factors, carries a better prognosis, and shows no familial history as depicted in fig 1.

2.1.2.2 Diffuse, infiltrative, endemic type gastric cancer

Diffuse gastric cancer or HDGC is a specific type of stomach cancer that tends to affect much of the stomach rather than staying in one area of the stomach as depicted in fig 2. This type is also not recognizably influenced by environment or diet, is more virulent in women, and occurs more often in relatively young patients. The average age for someone with HDGC to be diagnosed with stomach cancer is 38. HDGC is a genetic condition, follows an autosomal dominant inheritance pattern, so has a chance

to be passed from generation to generation in a family. A mutation in *CHD1* gene gives a person an increased risk of developing HDGC and is estimated to be about 65% for men and 80% for women, besides increases risk for developing other cancers like lobular breast cancer in women (Becker *et al*., 1994). It shows scattered cell clusters with poor differentiation and dangerously deceptive margins.

2.1.3 Pathology

Pathology of tumor is usually reported from the analysis of tissue taken from a biopsy or surgery. A pathology report will usually contain a description of cell type and grade.

2.1.4 Staging

Staging is a careful attempt to find out whether the cancer has spread and, if so, to what parts of the body and how much. It also helps in predicting a patients outlook (prognosis). Two main ways are the TNM (Tumors/nodes/metastases) system, from the American Joint Committee on cancer (AJCC staging manual, 2002) as shown in Table 1 and the number system.

Cancer of the stomach can spread directly, via lymphatics, or hematogenously. Direct extension into the omenta, pancreas, diaphragm, transverse colon or mesocolon, and duodenum is common. If the lesion extends beyond the gastric wall to a free peritoneal (i.e, serosal) surface, then peritoneal involvement is frequent. Hematogenous spread commonly results in liver metastasis. Gastric cancer can spread almost anywhere in the body, including the liver, lungs, brain, and bones.

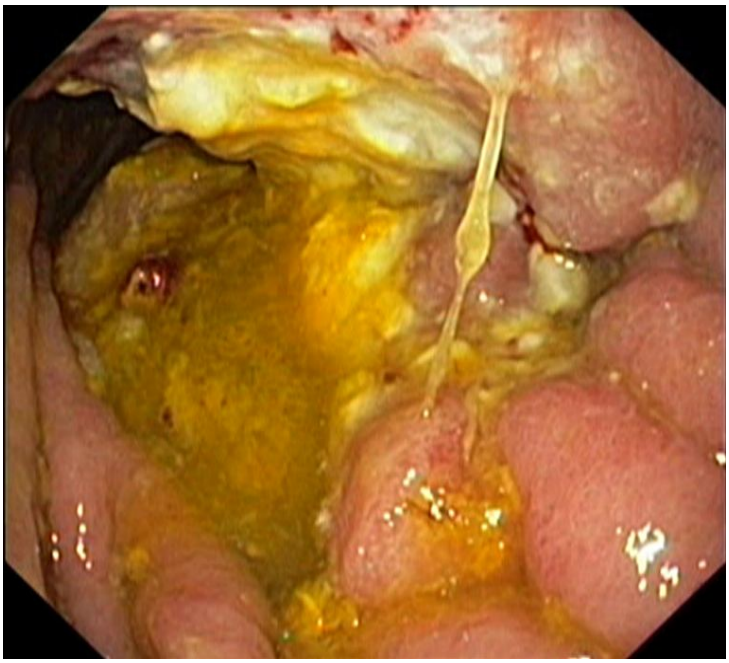

Fig 1: Adenocarcinoma of intestinal type

(Source: Wikipedia)

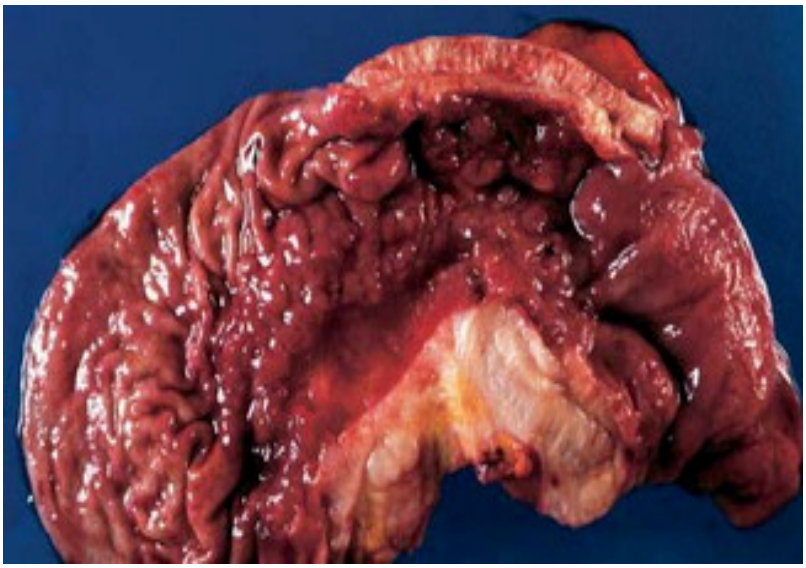

Fig 2: Gastric carcinoma of the diffuse type

(Source: Wikipedia)

Table1: TNM system of staging (AJCC staging manual,2002)

AJCC Stage	TNM stage	TNM stage criteria for gastric cancer
Stage 0	Tis N0 M0	Tis: Tumour confined to mucosa; cancer-in-situ
Stage I-A	T1 N0 M0	T1: Tumour invades submucosa
Stage I-B	T1 N1 M0	N1:Metastasis to 1-2 regional lymph nodes
	T2N0M0	T2:Tumor invades muscularis propia
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	T1-2N1 M0	N1: Metastasis to 1 to 3 regional lymph nodes. T1 or T2.
Stage III-B	T3-4N1 M0	N1: Metastasis to 1 to 3 regional lymph nodes. T3 or T4.
Stage III-C	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.

(Source: Wikipedia)

2.1.5 Incidence:

Cancer is a major burden worldwide but there are marked geographical variations in frequency and overall incidence. In 2008 approximately 12.7 million cancers were diagnosed (excluding non-melanoma skin cancers and other non-invasive cancers) and 7.6 million people died of cancer worldwide (Jemal, 2011). Gastric carcinoma is one of the most common cancer occurring globally (Parkin *et al.,* 2005) and second major cause of cancer related deaths in India (Peter and Bernard, 2008). The incidence of gastric cancer has decreased considerably in US over the past 60 years (Devesa *et al*.,1978). Incident rates are high in Japan, China, Chile and Ireland (Dunham *et al*., 1968). New estimated gastric cancer cases are: 21,320 (13,020 men and 8300 women) and Deaths: 10540 in US in 2012 (American cancer society: Cancer facts and figures 2012). Globally the highest rate in males was seen in Japan-80/100,000 and the lowest rate in Thailand-3/100,000. Among females also the highest rate of 31/100,000 was in Japan and lowest of 1 in Trivandrum, India (Curado *et al*., 2007). In India the high incidence areas includes North-East India (Phukan *et al*., 2001) and Kashmir valley (Khuroo *et al*.,1992), where environment and dietary habits play an overwhelming role in the development of stomach cancer over the genetic factors. Kashmir is a very high risk area of most commonly occurring cancers particularly cancers of gastrointestinal tract which comprise more than half the frequency of all the cancers (Shah and Jan, 1990; Mir *et al.,* 2005). In Kashmir, stomach cancer is the leading one with an average frequency of 19.2 % followed by esophagus and lung as 16.5 % and 14.6 %, respectively. Stomach (23 %) and lung (21 %) are the leading cancers in men while as esophageal cancer tops (18.3 %) in women followed by breast cancer (16.6 %) according to statistics obtained from a period of 5 years (Jan 2005 to Apr 2010) (Pandith and Siddiqui , 2012). Incidence of gastric cancer in Kashmir is three to six times higher than in other states (Khuroo *et al*., 1992). Almost two thirds of people with stomach cancer are 65 or older. The risk of a person developing stomach cancer in their lifetime is about 1 in 114, but is slightly higher in men than in woman with the ratio of 3.6:1 (Azra and Jan, 1990).

2.1.6 Risk Factors

Cancer is a multifactorial disease so it is hard to explain why one man develops cancer and another does not. However, we do know that person with certain risk factors

may be more likely than others to develop stomach cancer (Levin and Dozois, 1991). Some factors that have been associated with gastric cancer are as follows

- Family history of gastric cancer: Family history is being looked at as a risk factor for stomach cancer. Brothers, sisters, and children, of people with stomach cancer have an increased risk of getting it themselves.

- *Helicobacter pylori* infection (a common bacteria that can also cause stomach ulcers).

H.P, carcinogen class I (IARC,1994), colonizes the gastric epithelium and causes a severe inflammatory reaction that depends on factors including host genetic susceptibility, immune response, age at the time of initial infection, and environmental and virulence factors such as (*cagA*) (Wu *et al*., 2003, Franco *et al*., 2008; Umit *et al*., 2009). The complex interactions among the different types of H. *pylori*, inflammation and genetic features of the host could promote a cascade of morphological events leading to gastric cancer (Correa, 2004).

The Epstein Barr virus (EBV) has also found to be associated with gastric carcinoma in at least 10% of cases (Takada *et al*., 2000), is more prevalent in Japan (19.3%) and Germany (18%) (Takada *et al*., 2000; Van Beek *et al*., 2004).

- Dietary Factors: A diet high in starchy foods, salted and smoked foods, increases the risk of stomach cancer (Sriamporn *et al*., 2002; Azra, 1995; Morson, 1995). Stomach cancer levels are very high in Japan where very salty pickled foods are popular. A preserved food and pickels may also increase risk of developing cancer. In Kashmir a lot of dietary features and life style are peculiar, e.g., consumption of hot salted tea, sun-dried vegetables of Brassica family (Hakh), pickled vegetables (Anchar), dried fish, red chilies, spice cakes etc. These food items have been found to contain substantial amount of N-nitroso compounds including N-nitrosopipecolic acid, mono and diamines of methane and ethane, with several unidentified nonvolatile N-nitroso compounds (Kumar *et al.*, 1992; Siddiqi *et al.*, 1992; Siddiqi *et al.*, 1998).
- Age: Stomach cancer becomes more common as people get older. Around 95 out of every 100 cases (95%) are diagnosed in people aged 55.

- History of other cancers: Statistically, men have a slightly increased risk of stomach cancer if they've had prostate, breast, bladder or testicular cancer. Women have an increased stomach cancer risk if they've had ovarian, breast or cervical cancer. Both sexes have an increased risk if they've had food pipe (esophageal) cancer, non melanoma skin, bowel, non Hodgkin's lymphoma or thyroid cancer.
- Reduced immunity: People with suppressed immune systems due to infection with HIV, AIDS, or drugs taken following an organ transplant, have double the risk of stomach cancer compared to other people. This may be because they have an increased risk of infection such as *Helicobacter pylori*.
- Hormone replacement therapy (HRT): Women who take hormone replacement therapy have a reduced risk of stomach cancer. But HRT increases the risk of some other types of cancer, including breast cancer.
- History of an adenomatous gastric polyp larger than 2 centimeters
- History of chronic atrophic gastritis
- History of pernicious anemia
- Anti inflammatory drugs: Studies showed that people who regularly take non steroidal anti inflammatory drugs appear to have a slightly lower risk of stomach cancer e.g: aspirin, ibuprofen or Nurofen.
- Smoking: Cigarette smoke contains many carcinogens. Smoking can increase the risk of stomach cancer. About 1 in 5 stomach cancers (20%) in the UK is thought to be caused by smoking. People who smoke have around twice the risk of developing stomach cancer compared to non smokers. The risk falls if you stop smoking. If smokers have HP infection, they may have more than 10 times the risk of non smokers without HP infection.

Besides these risk factors, cancer can arise due to cumulative effect of mutations in various regulatory genes, or from epigenetic changes in DNA (Fearon *et al*., 1990; Vogelstein *et al*., 1988; Mustafa *et al*., 2007).

2.1.7 Epigenetics

Epigenetics has been found to be major concern for all type of cancers. Epigenetics can be described as a stable alteration in gene expression potential that takes place during development and cell proliferation, without any change in gene

sequence. This change, though heritable, is reversible, making it a therapeutic target. Recent studies have shown that epigenetics plays an important role in viral infections, (Baylin**,** 1997) cancer biology (Singal and Ginder, 1999; Jones and Baylin, 2002) activity of mobile elements, (Costello and Plass, 2001) somatic gene therapy, cloning, transgenic technologies, genomic imprinting, developmental abnormalities, mental health, and X-inactivation (Amir *et al.,* 1993; Laird, 2003). Epigenetic changes may involve DNA methylation, Histone acetylation, etc. DNA methylation is one of the most common epigenetic change. DNA methylation is a covalent chemical modification, resulting in the addition of a methyl (CH_3) group at the carbon 5 position of the cytosine ring. Even though most cytosine methylations occur in the sequence context 5'CG 3' (also called the CpG dinucleotide), some involves CpA and CpT dinucleotides (Ramsahoye *et al*., 2000). The human genome contains regions of unmethylated segments interspersed by methylated ones (Antequera and Bird, 1993). Approximately half of all the genes (housekeeping genes and genes with tissue specific patterns of expression) in humans have CpG islands (Bird, 1986; Singal and Ginder, 1999). DNA methylation is brought about by a group of enzymes known as the DNA methyltransferases (DNMTs). The DNMTs known to date are DNMT1, DNMT1b, DNMT1o, DNMT1p, DNMT2, DNMT3A, and DNMT3b with its isoforms, and DNMT3L (Robertson, 2002). DNMT1 has de novo as well as maintenance methyltransferase activity, and DNMT3A and DNMT3b are powerful de novo methyltransferases (Costello and Plass, 2001). The methylation profile of the cell is exquisitely controlled during development. Methylation patterns are established in the early embryo with initial demethylation of the parental DNA in the first few cell divisions after fertilization, followed by de novo methylation of specific CpG sites between the eight cell stage and blastocyst implantation (Monk, 1990; Howlett and Reik, 1991; Mayer *et al*.,2000; Reik *et al*., 2001). An apparent interplay between de novo methylation and demethylation at each cell division gives rise to a heterogenous pattern of methylation for any one molecule (Warnecke *et al*., 1998; Warnecke and Clark, 1999), so it has become clear that the methylation state of any one CpG site is not always maintained. The other machinery of methylation includes demethylases, methylation centers triggering DNA methylation, and methylation protection centers (Costello and Plass, 2001; Szyf, 2003).

DNA methylation in mammals is thought to be important for gene regulation control. Methylation within gene regulatory regions such as promoters and enhancers generally affects several important signaling pathways that are frequently activated in cancer cells by suppressing their function. For example, most promoter regions that are methylated *in vitro*, either from tissue-specific or CpG island associated genes, show reduced expression after transfection. Methylation induced suppression is thought to occur either by the blocking of transcription factor binding (Iguchi and Schaffner, 1989; Molloy and Watt, 1990) and/or by formation of an inactive chromatin state by histone modification (Nan *et al*., 1998; Bird and Wolffe, 1999; Magdinier and Wolffe, 2001). However, it is still unclear whether methylation directly elicits gene inactivation or is a consequence of gene silencing. For example, CpG islands on the inactive X chromosome are methylated subsequent to gene silencing. Several mechanisms have been proposed to account for transcriptional repression by DNA methylation. The first mechanism involves direct interference with the binding of specific transcription factors to their recognition sites in their respective promoters. Several transcription factors, including AP-2, c-Myc/Myn, the cyclic AMP-dependent activator CREB, E2F, and NFkB, recognize sequences that contain CpG residues, and binding of each has been shown to be inhibited by methylation. (Tate and Bird, 1993; Singal and Ginder, 1999).

The second mode of repression involves a direct binding of specific transcriptional repressors to methylated DNA or by directly preventing binding of transcriptional activators to DNA. The DNA methylation signals are analyzed by the MBDs, the target being the 5' methylated CpG sequence (Singal *et al*., 1997; Singal *et al*., 2001 and Prokhortchouk and Hendrich, 2002). MeCP1 and MeCP2 were the first two protein complexes identified (Klose and Bird, 2006).These contain MBD and TRD capable of silencing transcription. The TRD domain interacts with SIN3A - a transcriptional corepressor, suggesting a link between transcriptional repression, histone deacetylation and DNA methylation. The details of mechanism of transcriptional silencing is shown in figure 3.

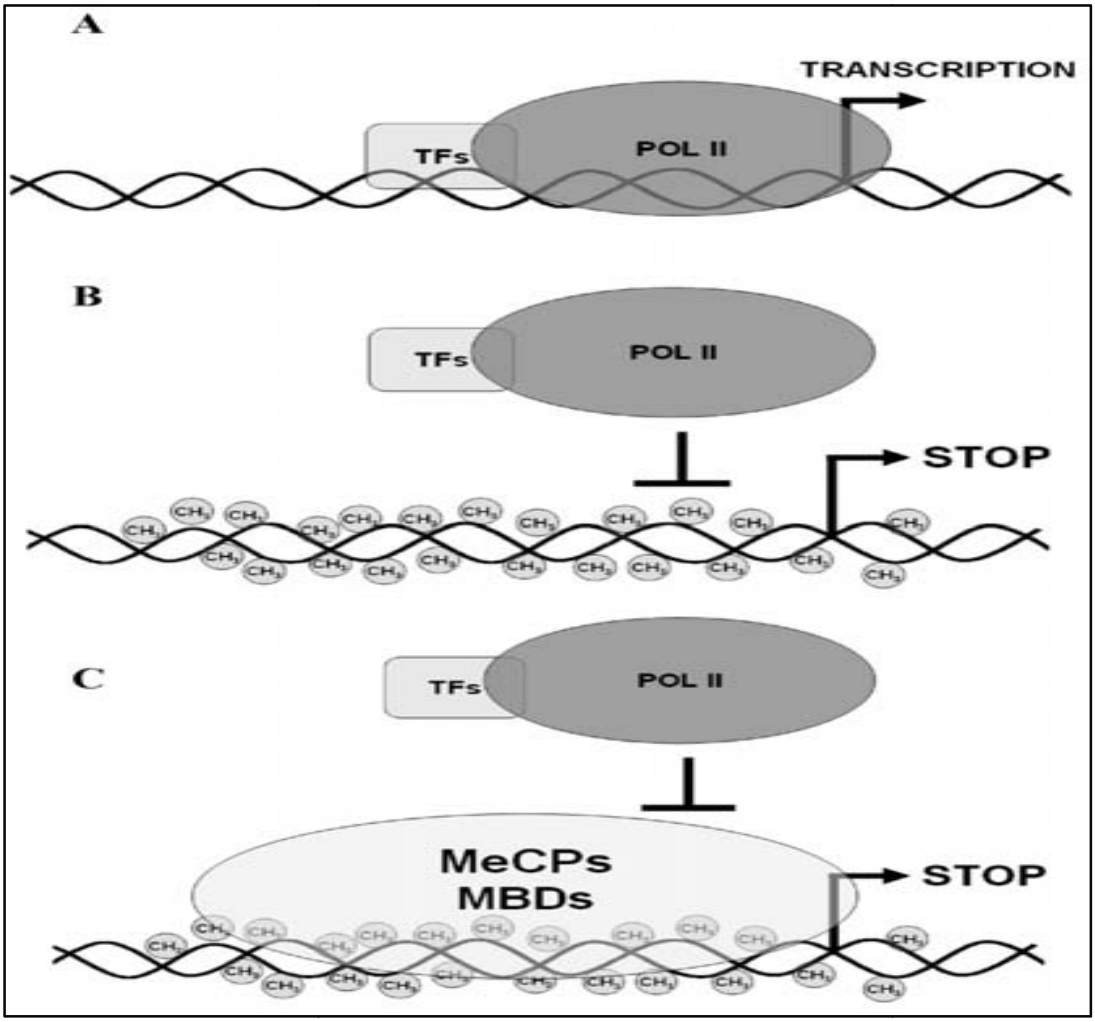

Figure 3. Repression of transcription via CpG dinucleotide methylation. Promoter sequence binds transctiption factors (TFs) and RNA polymerase II (POL II) that initiates transcription (A). Methylation of CpG within promoter binding site directly inhibits requirement of TFs and represses transcription (B). Methylated DNA binds m5CpG binding (MeCPs) and (MBDs) forming spatial obstacle that prevents binding of TFs to promoter sequence.

(Source :Wikipidea)

Hypermethylation in cancer reports far outnumber the reports of hypomethylation in cancer. The first tumor suppressor gene found to be silenced through promoter hypermethylation was *Rb1* (Sakai *et al*., 1991). DNA hypermethylation is considered as frequent event as mutations occurring within the coding region of these genes. Active transcription, active demethylation, replication timing, and local chromatin structure prevents access to the DNA methyltransferase and thus acts as protective mechanisms to prevent hypermethylation of the CpG islands (Clark and Melki, 2002). The genes that are susceptible are the genes involved in cell cycle regulation (*p16INK4a, p15INK4a, Rb, p14ARF*) genes associated with DNA repair (*BRCA1, MGMT*), apoptosis (*DAPK, TMS1*), drug resistance, detoxification, differentiation, angiogenesis, and metastasis. *GSTP1* gene, is found to be hypermethylated in more than 90% of prostate cancers but is largely unmethylated in acute myeloid leukemia. (Lee *et al*.,1994; Melki *et al*., 1999). The mechanisms involved in targeting of methylation to specific genes in cancer remain to be determined.

Besides hypermethylation, hypomethylation is also observed in a wide variety of malignancies (Feinberg and Vogelstein, 1983; Kim *et al*., 1994). Genome wide hypomethylation is believed to cause inappropriate proto-oncogene activation and transcription, and malignant transformation (Jones and Baylin, 2002; Feinberg and Tycko, 2004). Hypomethylation of CpGs was reported in colorectal cancers by Fearon *et al*. in 1983 (Feinberg and Vogelstein, 1983). It is common in solid tumors such as metastatic hepatocellular cancer, (Lin *et al*., 2001) in cervical cancer, (Kim *et al*., 1994) prostate tumors, (Bedford and Helden, 1987) and also in hematologic malignancies such as B-cell chronic lymhocytic leukemia (Ehrlich, 2002). Hypomethylation of retrotransposons causes transcriptional activation and has been found in many types of cancer, such as urinary bladder cancer (Jurgens *et al*., 1996). Inadequate dietary folate has been implicated in the development of several types of cancers (Duthie *et al*., 2004). One proposed mechanism is that folate deficiency might induce DNA hypomethylation. High alcohol intake reduces intracellular levels of SAM thus causes DNA hypomethylation, besides cleaves folate, impair folate absorption and increases folate excretion (Kenyon *et al.,* 1998).

The detection of epigenetic alteration in tumorigenesis has led to a host of innovative diagnostic and therapeutic strategies. Epigenetic changes have been detected in the body fluids of almost every organ system in cancer patients (Laird**,** 2003). This would thus help us to know patients response to treatment and predicting survival. *CDKN2A* gene was found to be hypermethylated in 61.1% of colon tumor samples, and this was correlated with the traditional prognostic indicators, such as tumor grading and Dukes' staging (Maeda *et al*., 2003). Similarly, *TCF4* was found to be hypermethylated in colon cancers and hypermethylation was found to be high in stage I/II than in stage III/IV in gastric cancer (Kim *et al*., 2008). For many epigenetically silenced genes, re-expression in tumor cells can lead to suppression of cell growth or altered sensitivity to existing anticancer therapies and small molecules that reverse epigenetic inactivation like demethylating drugs are now undergoing clinical trials in cancer patients (Momparler *et al*., 1997; Pohlmann *et al*., 2002) to reverse the silencing of genes resulting from methylation (Strathdee *et al.,* 1999; Plumb *et al.,* 2000) Thus, epigenetic alterations are not only potential therapeutic targets because of their reversibility, but also potential biomarkers that can be used to detect and diagnose cancer in its earliest stages (Brown *et al*., 2002). This potential to reverse DNA methylation and re-express the affected critical genes presents an attractive option for exploring clinical use in malignancies. The commonly used drugs targeting methylation are azacytidine (5-azacytidine), decitabine (5-aza-2-deoxycytidine), fazarabine (1-D-arabinofurasonyl-5-azacytosine), and dihydro-5 azacytidine (Goffin and Eisenhauer, 2002). HDAC inhibitors are also being tried as potential chemotherapeutic agents (Thiagalingam *et al*., 2003) as DNA methylation represses gene expression in part through histone deacetylation, HDAC inhibitors have been used to activate expression from methylated genes but these work together with demethylating agents (Cameron *et al.,* 1999). Preclinical studies on decitabine have shown that it reverses methylation in a number of cell lines and in cells from human leukemia patients (Wilson *et al.,* 1983; Momparler *et al*., 1984).

2.1.8 TCF4 Gene

TCF4

The Transcription factor 4 also known by other names:

- HLHb19
- Class B basic helix-loop-helix protein 19
- E2-2
- ITF2
- SEF2

The tcf4 protein is encoded by *TCF4* gene. Molecular Location on chromosome 18: base pairs 52,889,561 to 53,255,859 on chromosome 18. It has 41 exons of which 21 are alternative 5' exons situated at various positions throughout the gene as depicted in figure 4.

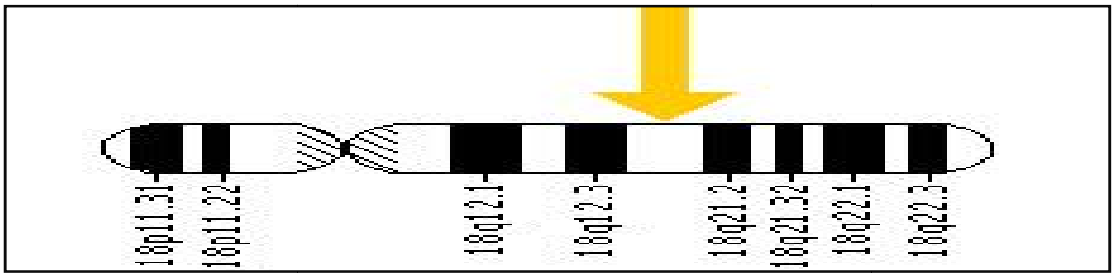

Fig 4: Cytogenetic Location: 18q21.1 (The *TCF4* gene is located on the long (q) arm of chromosome 18 at position 21.1).

(Source Wikipedia)

The *tcf4* protein shows its expression before birth in various tissues. It plays a role in the maturation of cells to carry out specific functions like cell differentiation and apoptosis. The lowest quantities of *tcf4* transcripts were present in fetal liver, pancreas and colon. The highest levels are present in fetal brain, but expression remains elevated also in adult brain and is required for adult tissue maintenance in bone, heart, muscle. Nevertheless, it seems that for production of sufficient amounts of *tcf4* protein and normal development, the presence of all transcription initiation sites are important.

The Transcription factor 4 gene product is a member of the class I basic helix-loop-helix (bHLH) family (Masaari *et al*., 2000). On the basis of biochemical and functional criteria , bHLH family of transcription factors is categorized into various classes and each member protein contains an HLH domain and a basic

DNA binding domain, at its N-terminal site to make contact with consensus DNA sequences known as E-boxes (CANNTG) , found in the promoters of various important genes , driving their specific activation (Church *et al*, 1985 ; Murre *et al*,1994; Saisanit and Sun, 1995; and Naya *et al*.,1995; Atchley and Fitch, 1997 and Ledent *et al*., 2002). E-proteins are critical regulators in a diverse array of biological processes such as cell growth, differentiation, tissue-specific gene expression, and programmed cell death (Pagliuca, 2000; Massari *et al*, 2000; Jones, 2004) ,these can form homodimers and heterodimers with other classes of bHLH proteins through the HLH domain to facilitate binding to DNA (Murre *et al*., 1989) and this dimerization regulates tissue-specific gene expression like differentiation and proliferation of myocytes (Lassar *et al*., 1991), osteoblasts (Beck *et al*., 2001), B and T lymphocytes (Quong *et al*., 2002), and neuronal cells (Persson *et al*.,2000**)** through E-box sites . These E-proteins function as transcription activators or repressors.

Exon 1 of *TCF4* has β –catenin binding domain, exon 10 and 11 has DNA binding HMG boxes and exon 17 has COOH terminal binding domain. Previous studies have presented controversial cellular roles for *TCF4* (Pagliuca *et al*., 2000 and Kolligs *et al*., 2002). It has also been established that *TCF4* silencing was more frequent in early stage gastric cancers than in advanced stage gastric cancers, besides its silencing is associated with cell growth and migration in gastric cancer cell line. *TCF4* is a end product of Wnt signaling pathway (Behrens *et al*., 1996; Korinek *et al*., 1997), and plays an important role in malignant transformation (Cadigan *et al*., 1997). c- myc, cyclin D1, c-Jun, MMP7 are targets for Wnt pathway. Mutations in this pathway in adults contribute to degenerative diseases and cancers. The β -catenin / TCF4 complex imposes a crypt progenitor phenotype on colorectal cancer cells and maintains the undifferentiated state of intestinal crypt progenitor cells (Van Es *et al*., 2005). Thus the β –catenin /*TCF4* constitutes the master switch that controls proliferation versus differentiation in healthy and malignant intestinal epithelial cells. The *TCF4* target gene c myc plays a central role in this switch by direct repression of p21 (CIP/WAF12 promoter) (Van de watering *et al*., 2002). It has also been shown that the enforced expression of *TCF4* suppresses the colony-forming efficiency of cells in several cell lines, suggesting its role as a negative regulator of cell proliferation (Pagliuca *et al.,* 2000). The loss of epithelial cell polarity may also contribute to intestinal tumorogenesis (Naishiro *et al*., 2001).

TCF4 is critical for nervous system functioning as its mutation causes PHS (Brockschmidt *et al*., 2007; Amiel *et al*., 2007 and Zweier *et al*., 2007), a neurodevelopmental disease characterized by mental retardation, seizures, and hyperventilation (Pitt and Hopkins., 1978 and Peippo *et al*.,2006) and also SNP of *TCF4* in exon 17 are observed in Renal cell carcinoma (Hiroaki Shiina *et al*., 2003).Mutation of *TCF4* is also found in gastric cancer and breast cancer (Burwinkel *et al*., 2006; Kim *et al*., 2009; Kojima *et al*.,2011). And these mutations is found to enhance cell growth in various cell lines like in Primary CRCs , so making it as a tumor suppressor gene (Sjoblom *et al*., 2006; Wood *et al*.,2007; Tang *et al*.,2008). However. the size of the mutation does not appear to affect the severity of the disease as people with large deletions and those with single nucleotide changes seem to have similar signs and symptoms. One study also revealed that silencing of *TCF4* caused significant sensitization of CRC cells to clinically relevant doses of X- rays (Kendziorra *et al*., 2011). *TCF4* gene's inability to bind to DNA and control the activity of genes involved in nervous system development and function, and genes like cyclin D1, c myc ,c jun, etc involved in cell cycle functions contributes significantly to the signs and symptoms of Pitt-Hopkins syndrome and significantly to carcinogenesis.

DNA methylation and modification of chromatin structure often occur in neoplasia. Aberrant methylation of CpG islands in the promoter regions and in the initial exons of many genes occurs in the early stages of carcinogenesis and results in suppressed expression of a variety of genes in a diverse array of cancers (Estellar, 2002; Herman and Baylin, 2003). Many reports have also shown that methylation of CpG islands of *TCF4* gene leads to its inactivation particularly in gastric cancer (Grady *et al*.,2000; Shim *et al.*, 2000 ; Iida, 2000; Oue *et al*, 2001 and Kim *et al*.,2006). *TCF4* is considered as age related as well as ca specific methylated gene (Type A & C) in Gastric cancer. Gastric cancer the second main cause of death in almost every country (Parkin *et al*., 2005), is not diagnosed until at an advanced stage. Therefore, identification of effective biomarkers for early-stage detection of gastric cancers is needed. In this study, the aberrant promoter hypermethylation of *TCF4* gene in gastric cancer was demonstrated. The result suggested that promoter hypermethylation of this gene plays an important role in gastric tumorigenesis.

CHAPTER:3

METHODOLOGY

A case-control study was undertaken to understand the etiology of gastric cancer in Kashmir valley: A state with high incidence of this dreadful disease. All ethical considerations were taken care of during the study and the recruitment process was started only after ethical clearance by the Departmental Ethical Committee as per norms. Subjects with histopathologically confirmed gastric carcinoma tissue samples and histopathologically confirmed gastric normal tissue samples were evaluated. Histopathologically confirmed gastric cancer tissue samples were cases while as histopathologically confirmed normal tissue samples were treated as controls. The samples were collected from Department of Surgery, Shri Maharaja Hari Singh Hospital associated with Government Medical College, Srinagar J&K. The sample size was 80 out of which 50 were cases and 30 controls. Record of complete case history of patients was maintained.

3.1 CASES

Samples of gastric cases patients that were operated in the Department of Surgery, S.M.H.S. Hospital, Srinagar, and Private administered Hospitals were included in the study. During the study, cases were included irrespective of their age and stage of the cancer.

3.1.1 Inclusion Criteria

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

- All histopathologically confirmed patients irrespective of cancer stage and age.
- Patients of Kashmiri origin.

3.1.2 Exclusion Criteria

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

- Patients suffering from any other disease
- Patients who had received prior chemo or radiotherapy
- Patients not of Kashmiri origin
- Any other type of cancer

3.2 CONTROLS

Resected gastric samples from the Department of Surgery, S.M.H.S. Hospital which were histopathologically confirmed as normal were processed as controls.

3.2.1 Inclusion criteria

- Patients of Kashmiri origin.

3.2.2 Exclusion criteria

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

3.3 COLLECTION OF TISSUE SAMPLES

The case and control samples were put in plastic vials (50 ml volume) and the vials were properly labeled, to avoid possible mixing of sample vials and for easy retrieval of the required sample vial. The collected samples were divided into two parts and one was kept in 10% formalin for histopathological evaluation and other part in normal saline and was kept at -80 ºC for further analysis. Histopathological report of all the collected samples were collected before they were further processed. Records were maintained carrying information regarding gender, age, history of disease, etc.

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} Mini Prep supplied by ZYMO RESEARCH. The protocol followed was as directed by the company.

The DNA extracted was stored at 4 º C for a short duration but the vials were kept at -20^{o}C for longer duration storage for further investigation.

3.4.2 QUALITATIVE AND QUANTITATIVE ANALYSIS OF GENOMIC DNA

3.4.2.1 Qualitative Analysis

The integrity of the genomic DNA was examined by gel electrophoresis using 1 % agarose gel to which 10µl/ 50ml (of gel solution) of fluorescent dye ethidium bromide was added during its cooling. The gel was cast and 20µl wells were made into it by using suitable combs. 2µl of each DNA sample was mixed with 1µl of 1X DNA loading dye (4.16 mg bromophenol blue, 4.16 mg xylene cyanol and 0.66g sucrose) and was loaded in the gel. Electric current was applied at 50 volt until DNA entered in to the gel and potential was raised to 70 volt for rest of the run. Run was stopped when the dye had travelled nearly two- third 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.

3.4.2.2 Quantitative Analysis

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

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

Dilution factor (D.F). = volume of final diluted solution/volume of original concentrated solution.

The purity of DNA was estimated by obtaining the ratio of values of absorbance at 260 and 280.

$$\text{Ratio} = A_{260} / A_{280}$$

The ratio of A_{260}/A_{280} was calculated and the DNA sample for which the ratio was 1.7-1.9 was considered suitable for the future use. DNA was alliqouted into three to four tubes so as to protect damage from freeze thawing and store at -20 $^{\circ}$C freezer for longer duration of time.

3.5 DNA MODIFICATION (BISULFITE TREATMENT)

The above extracted Genomic DNA was modified by EZ DNA Methylation–Direct™ Kit supplied by ZYMO RESEARCH. The protocol followed was as directed by the company.

Sodium bisulfite treatment converted unmethylated cytosines to uracil as shown in figure 5. DNA, however, remains unmodified at places where DNA was methylated. This modification can help us differentiate between methylated and unmethylated DNA using specific primers in MS-PCR. Now DNA can be subjected to immediate analysis or can be stored at or below -20°C for later use.

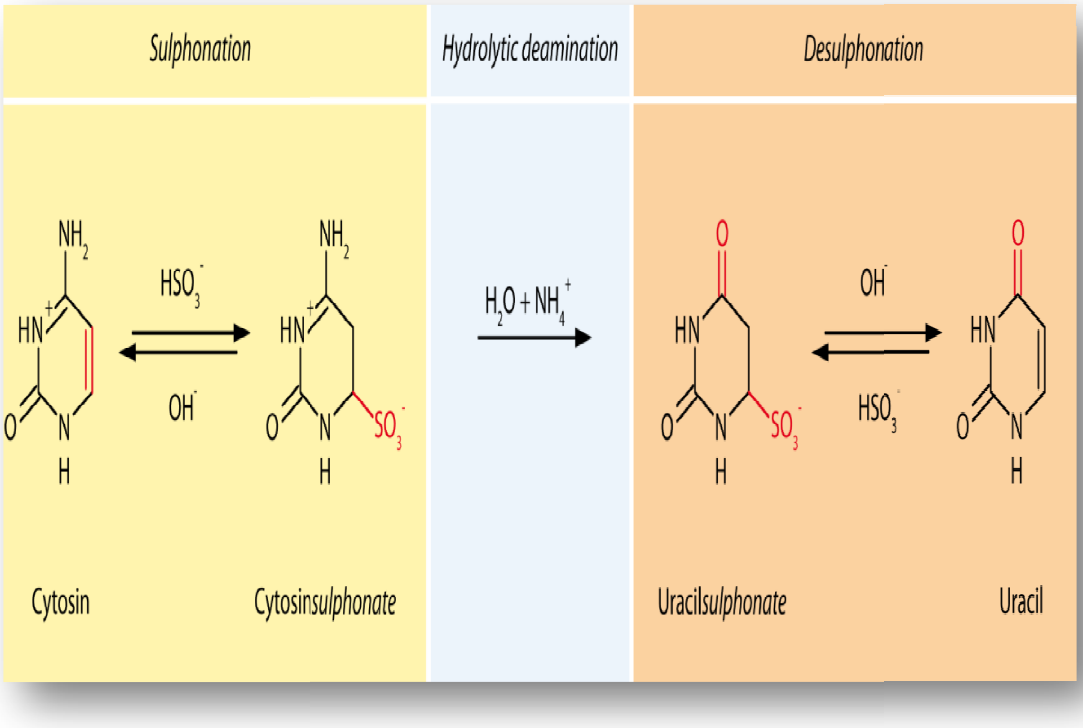

Fig. 5: Diagrammatic representation of cytosine conversion to uracil

3.6 METHYL SPECFIC POLYMERASE CHAIN REACTION (MSP)

To determine the status of *TCF4* promoter methylation in gastric cases from Kashmir valley, Methyl Specific PCR (MSP) was performed for a promoter region of *TCF4* gene in 50 surgically resected gastric cancer DNA and compared with that of 30 histopathologically confirmed normal gastric tissues. MSP is a novel and sensitive way for detection of hypermethylation in CpG islands of DNA.

The principle of this PCR method lies in the amplification of the hypermethylated and non-methylated DNA of the same gene by different primer

sequence; one for hypermethylated version of the gene and one for the non-methylated version of the same gene. Table 2 shows unmethylated and methylated sets of primers of *TCF4* gene. Thus by visualising the PCR product we can easily determine whether amplification is by hypermethylated or non-methylated primers, thus determine whether our CpG's were hypermethylated or unmethylated. The methylated cytosine pairs with guanine and unmethylated modified to uracil (C converted to U) pairs with A during annealing.

The modified DNA was taken into two PCR vials in equal quantity and same amount of all reagents (Table 3) was added to both the vials but in one vial methylated primers were used and in second vial non-methylated primers were used.

PCR amplification was achieved using a Thermal cycler (Gradient thermal cycler from (EPPENDORF MASTERCYCLER PRO). Reactions were hot-started at 95ºC for 5 min, followed by addition of T*aq* Polymerase, followed by 35 cycles of melting (95ºC for 45 sec.), annealing (59ºC for 45 sec.) and extension (72ºC for 45 sec.) and by final extension step at 72º C for 4 min (Table 4).

Universal Methylated Human DNA Standard and Control with primers (ZYMO RESEARCH) was used as positive control, and water was used as negative control.
Each PCR reaction (10 µl) was directly loaded onto non denaturing 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination.
The amplified DNA were of approximately same base pairs in length, the methylated and the unmethylated PCR product were of 258 bp and 259bp and were then visualized under UV light in presence of a 100 bp DNA ladder run parallel to the amplified PCR products on 2% ethidium bromide pre-loaded agarose gel.

3.7 STATISTICAL ANALYSIS

The χ^2-test with Odds ratio was used to examine the association between hypermethylation of *TCF4* gene and cancer in gastric samples in a case-control study. 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.

Table 2: The DNA sample was amplified using the following primer pairs, two for each gene (Kim *et al.*, 2008).

Nature of Sequence	**Primer sequence**	
UNMETHYLATED PRIMER	Forward primer	5'- TGA ATT TGT STTT GTG TGT TTT T G-3'
	Reverse primer	5'- AAA AAA AAC TCT CCA TAC ACCACC-3'
METHYLATED PRIMER	Forward primer	5'- GAA TTT GTA ATT TCG TGC GTT TC-3'
	Reverse primer	5'- AAA AAA AAC TCT CCG TAC ACC G-3'

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

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

Table 4: Thermal cycling conditions

Steps	Temperature^{0}C	Time	Number of cycles
1. Hot-Start	95	5 min	1
2. Denaturation	95	45 sec	35
3. Annealing	59-61	45 sec	
4. Extension	72	45 sec	
5. Final extension	72	4 min	1

CHAPTER: 4

RESULTS

4.1 Methylation status of *TCF4* gene in cases and control

4.1.1 Cases

In the present study 50 histopathologically confirmed gastric cancer cases belonging to Kashmir division were analyzed for promoter region hypermethylation of *TCF4* gene. Out of 50 cases 33 were males which correspond to 66% and remaining 17 were females which correspond to 34%. The patients of gastric cancer belonged to different regions of Kashmir valley. Most often cancer was diagnosed at a stage when the disease was less likely to be cured.

4.1.2 Controls

Thirty histopathologically confirmed normal gastric cancer tissues were analyzed and taken as controls. Out of 30 normal cases 15 were males and remaining 15 cases were females.

4.1.3 Extraction of genomic DNA

Genomic DNA was isolated by kit method. Genomic DNA was isolated from all 80 samples (50 cases and 30 controls)

4.1.4 Qualitative analysis

The integrity of the genomic DNA isolated from tissue samples was examined on 1% agarose gel. Representative gel picture is given in figure 6.

4.1.5 Quantitative analysis

Quantity of the DNA was determined by using double beam spectrophotometer (Evolution 60 S from Thermo Scientific) and following equation is used to determine concentration

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

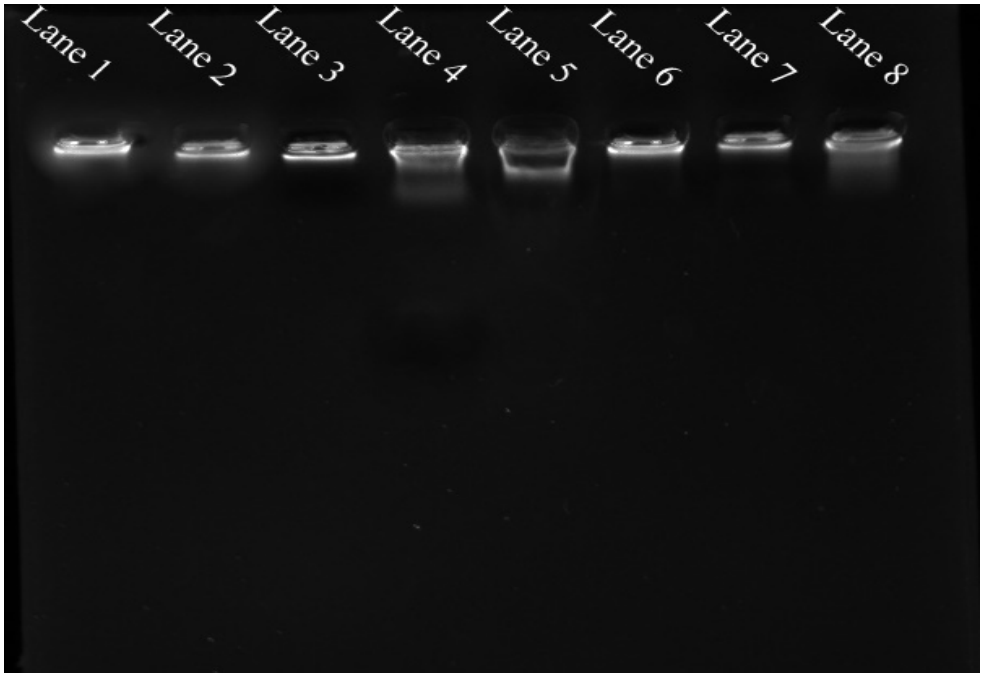

Fig 6: Representative gel picture showing the integrity of the genomic DNA on 1% agarose gel Lane 1 to 4 contains the genomic DNA isolated from the tissue samples of gastric cancer cases (GC1 to GC 4) and Lane 4 to 8 contains the genomic DNA isolated from the tissue samples of gastric normals (controls) (N1 to N4)

4.2 ANALYSIS OF *TCF4* GENE PROMOTER HYPERMETHYLATION IN CASES AND CONTROLS

To determine the status of *TCF4* promoter hypermethylation in gastric cancer cases from Kashmir valley, the MS-PCR for the promoter region (exon 1) of *TCF4* gene in 50 surgically Resected gastric cancer DNA was performed and compared with that of 30 histopathologically confirmed normal gastric tissues. Primers described (Kim *et al*., 2008) were used to discriminate between methylated and unmethylated DNA following bisulfite treatment. The amplicons were analysed on 2% agarose gel. Amplification was carried out using hot start PCR method, this decreases the non specific amplifications. The methylated and unmethylated PCR products were of **258** and **259** bp respectively. Gastric cancer samples were amplified by using both methylated and unmethylated set of primers in MS- PCR as shown in fig7, but cases were found to be amplified by methylated primers only, indicating that gastric cancer cases show *TCF4* promoter hypermethylation. Similarly, histopathologically confirmed normals were amplified by using both types of primers i.e., methylated and unmethylated ones as shown in fig 8, but the samples were found to be amplified by

unmethylated primers only, indicating that normal samples do not show promoter hypermethylation of *TCF4* gene. In addition, fig 9, shows normal samples amplified by unmethylated set of primers only, further indicating that normals do not show promoter hypermethylation.

As far as the frequency is concerned, 66% (33/50) of the gastric cancer tissues were found to have methylated *TCF4* promoter and 34% (17/50) of the cases had unmethylated *TCF4* promoter. The data depicted in table 5. In normal gastric tissues, 80% (24/30) of the normals were found to have unmethylated promoter and 20% (6/30) had methylated one. The results are given in table 6.

The association of promoter hypermethylation with gastric cancer was evaluated using Chi-square test and was found to be significant (p=0.0002, Odds ratio = 7.765, 95% C.I= 2.66- 22. 62) as depicted in fig 10.

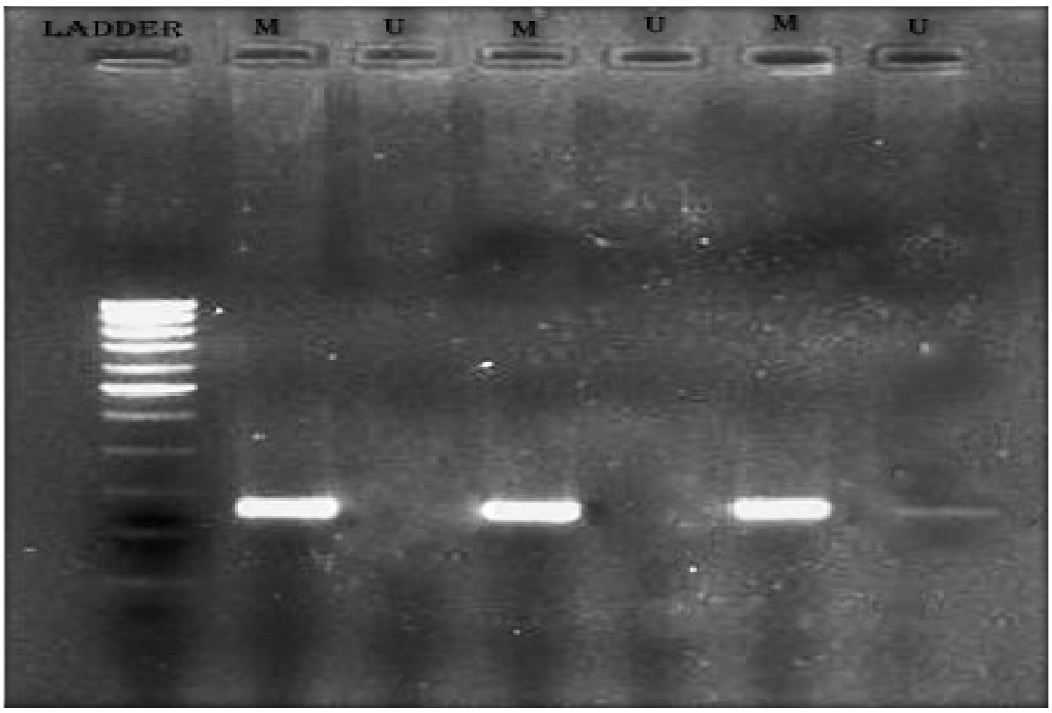

Figure 7: Representing MSP (Methylation Specific PCR) of Gastric cancer DNA samples run on 2% agarose gel. Product sizes: *TCF4* Unmethylated, 259 bp; *TCF4* Methylated, 258 bp

Ladder; 100 bp

M- Represents methylated product; U-Represents unmethylated product.

Lane 2, 4, 6: represents gastric cancer samples (G4, G9, G18) amplified by methylated primers

Lane 3, 5, 7: represents gastric cancer samples (G4, G9, G18) amplified by unmethylated primers

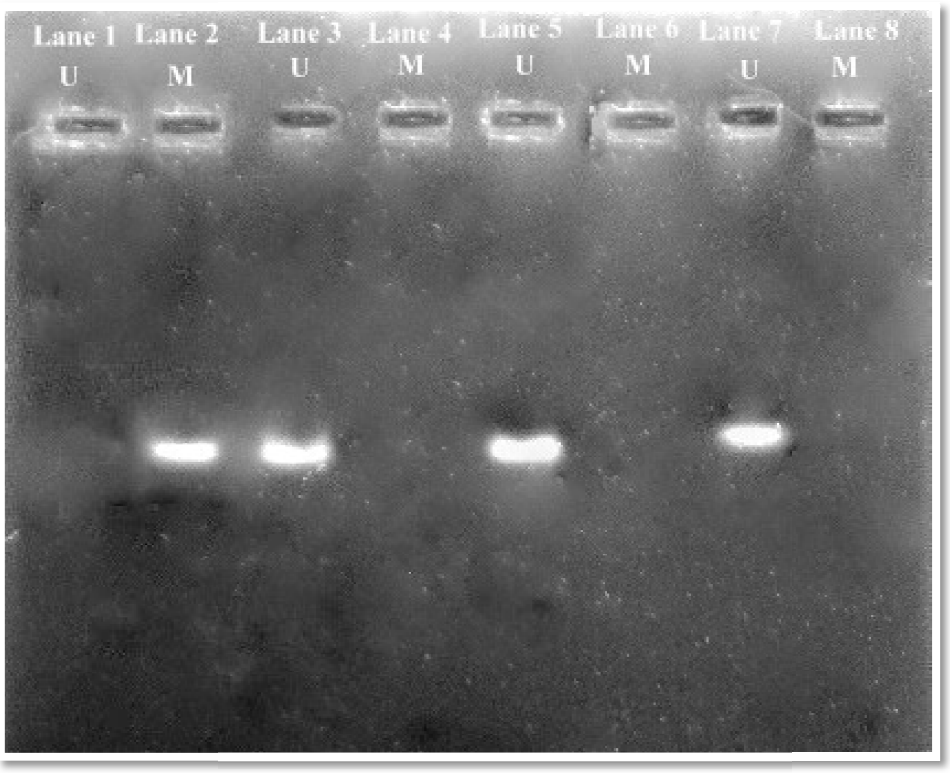

Figure 8: Representing MSP (Methylation Specific PCR) of histopathologically confirmed normal gastric controls run on 2% agarose gel. MSP was performed with primers specific for Methylated (M) and Unmethylated (U) regions.

Lanes 1: Represents negative control

Lanes 2: Represents positive methylated control

Lane 3, 5, 7: represents normal samples (N6, N10 and N20) amplified by unmethylated primers only

Lane 4, 6, 8: represents samples (N6, N10, N20) amplified by methylated ones.

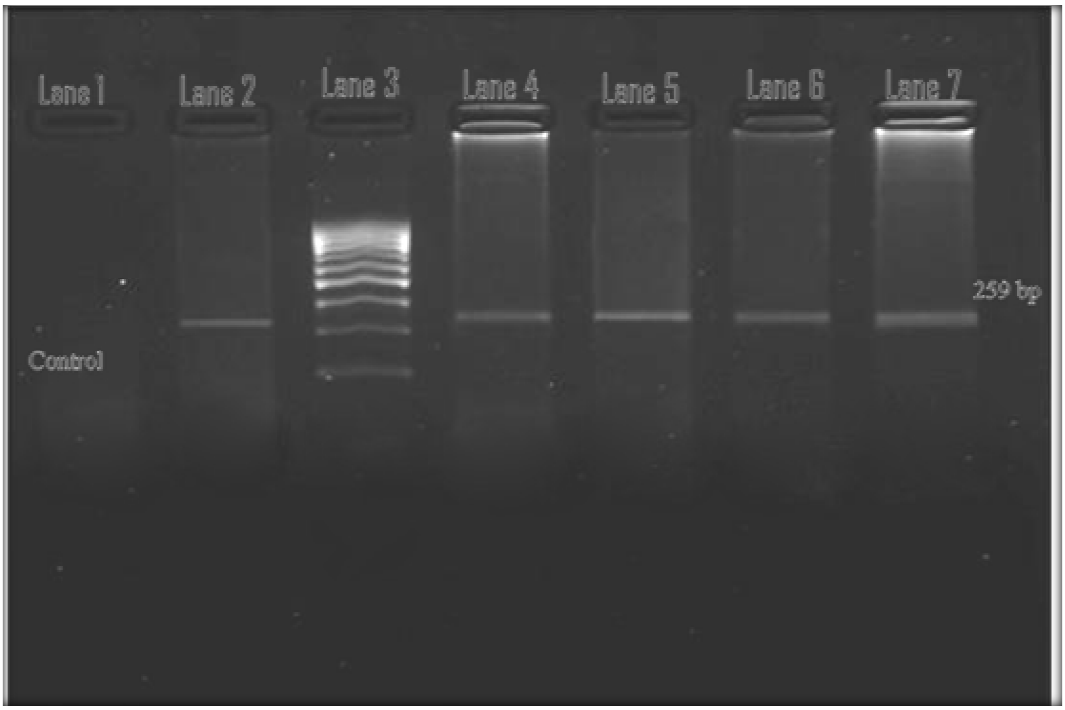

Figure 9: Representing MSP (Methylation Specific PCR) of normal gastric control DNA samples run on 2% agarose gel amplified by unmethylated primers only.

Lane 1- Represents Negative control

Lane 2- Represents Positive control (Universal methylated human DNA)

Lane 3- Represents 100 Bp ladder

Lane 4, 5, 6 and 7- Represents normal samples –N1, N7, N14, and N21; amplified by unmethylated primers only.

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

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

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

CONTROLS (30)		
PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATED	6	20% (6/30)
NON HYPERMETHYLATED	24	80% (24/30)

Odds ratio = 7.765, 95% C.I= 2.66-22.62, **p = 0.0002**

(Statistically significant p<0.05)

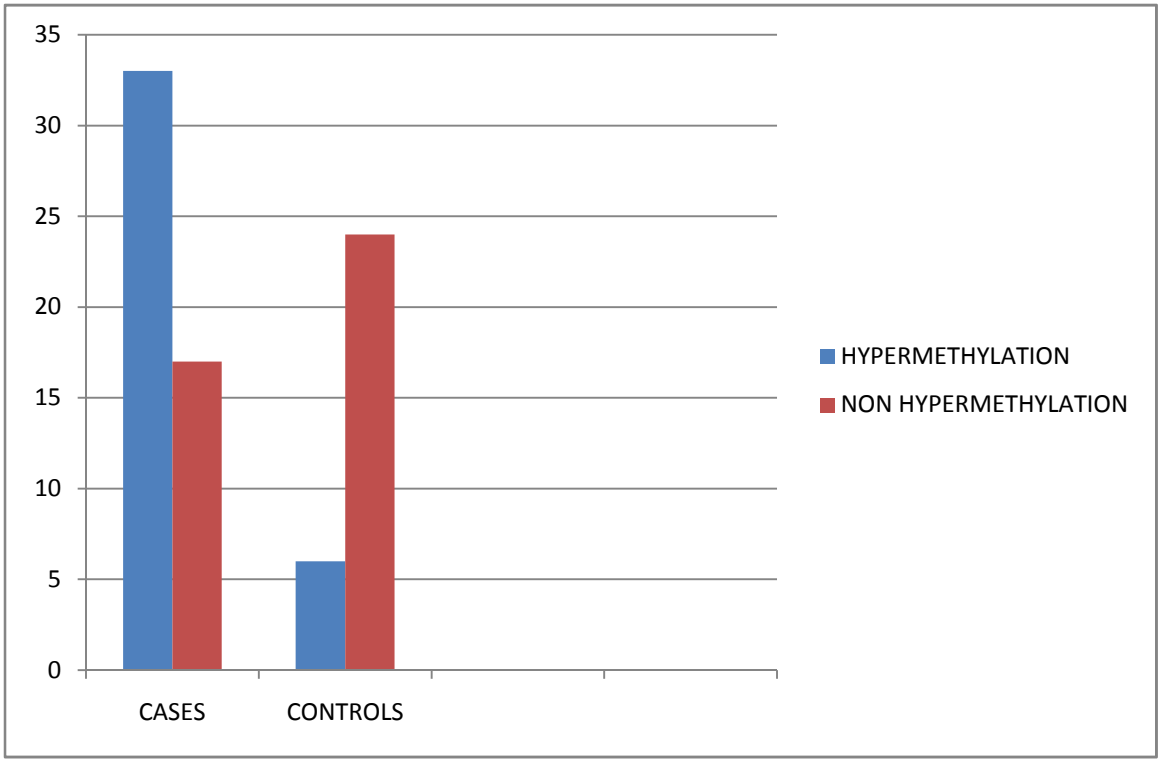

Fig 10: Histogram representing hypermethylated and non hypermethylated cases of gastric cancer and histopathologically confirmed normal controls

Odds ratio = 7.765,

95% C.I= 2.66-22.62

p = 0.0002 (Statistically significant p<0.05)

4.3 Relationship between promoter hypermethylation of *TCF4* gene and in males and females

Occurrence of *TCF4* methylation was found to be unequally distributed in males and females with more frequency in males than in females. Among 33 males, 22 cases were hypermethylated and 11 were unhypermethylated and among 15 male controls, 4 cases were hypermethylated and 11 were unhypermethylated shown in Table no 7. The association of promoter hypermethylation with gastric cancer was evaluated using Fisher's exact test and was found to be significant in males (O.D=5.5, 95% C,I=1.4-21.31 p=0.023),shown in fig 11. In comparison, among 17 females, 11 cases were hypermethylated and 6 Cases were unhypermethylated and among 15 females controls 3 Cases were hypermethylated and 12 Cases were unhypermethylated as shown in Table 8. The association of promoter hypermethylation with gastric cancer was evaluated using Fisher's exact test and was found to be significant in females too (O.D=7.33, 95% C,I=1.4-36.68 p=0.0287) shown in figure 12 .However on comparing the male cases with female cases, 22 cases were hypermethylated and 11 Cases were unhypermethylated in males and 11 cases were hypermethylated and 6 cases were found to be unhypermethylated in females (Table 9),occurrence of *TCF4* was found to be unequally distributed in males than in females but the difference was not statistically significant(O.D=0.916,95% C,I=0.26- 3.31,p=0.08599),shown in figure 13.The hypermethylation status of *TCF4* in males 66.66% (22/33) was also found to be higher than females 64.70% (11/17) in cases.

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

MALES CASES (33)

PARAMETER	CASES	FREQUENCY
HYPERMETHYLATED	22	66.66% (22/33)
NON HYPERMETHYLATED	11	33.33% (11/33)

MALE CONTROLS (15)

PARAMETER	CONTROLS	FREQUENCY
HYPERMETHYLATED	4	26.66% (4/15)
NON HYPERMETHYLATED	11	73.33% (11/15)

Odds ratio = 5.5, 95% C.I= 1.4-21.31, **p = 0.0023**

(Statistically significant p<0.05)

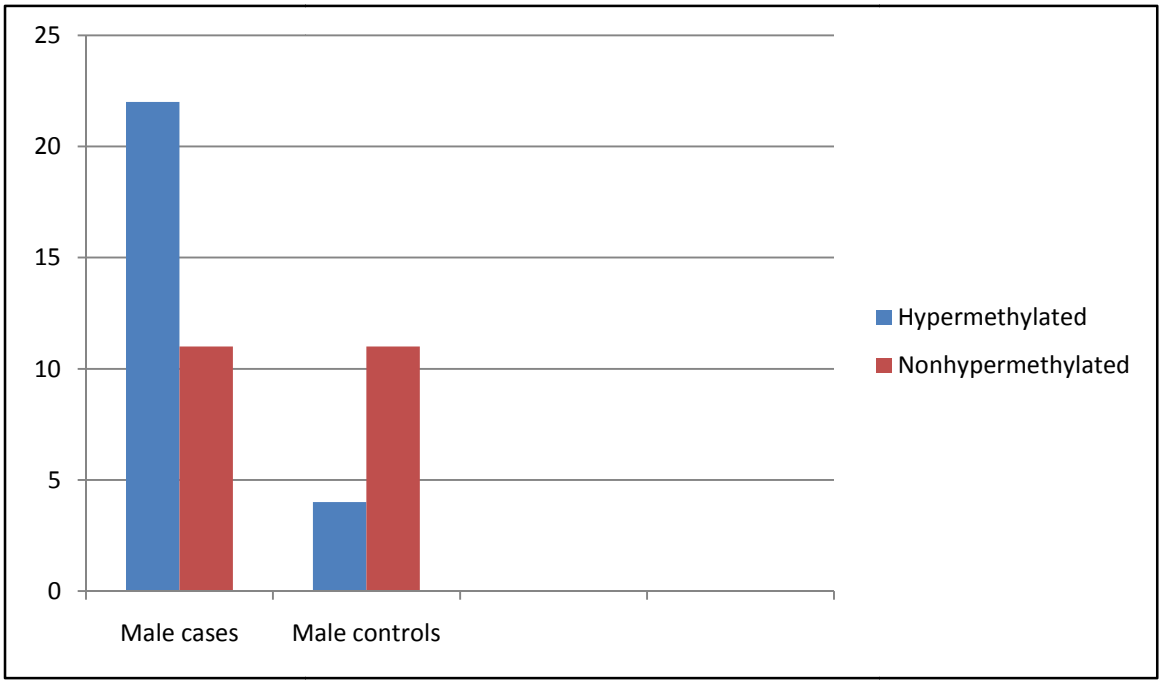

Fig 11: Histogram representing hypermethylated and nonhypermethylated male gastric cancer cases and histopathologically confirmed normal male cases.

O.D=5.5,

95% C.I= 1.4-21.31

p = 0.023 (Statistically significant p<0.05)

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

FEMALE CASES (17)		
PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATED	11	64.70 % (11/17)
NON HYPERMETHYLATED	6	35.29% (6/17)

FEMALE CONTROLS (15)		
PARAMETER		**CONTROLS FREQUENCY**
HYPERMETHYLATED	3	20% (3/15)
NON HYPERMETHYLATED	12	80% (12/15)

Odds ratio = 7.33, 95% C.I= 1.4-36.68, **p = 0.0287**

(Statistically significant p<0.05)

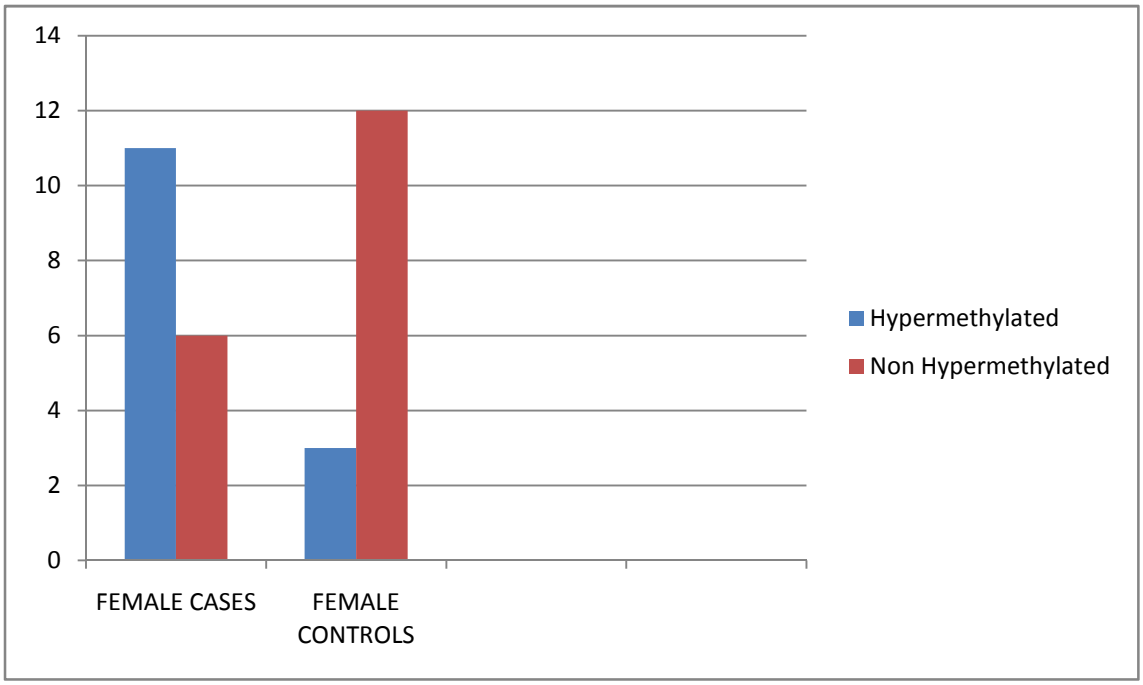

Fig 12: Histogram representing hypermethylated and non hypermethylated female gastric cancer cases and histopathologically confirmed normal female cases.

O.D=7.33,

95% C.I=1.4-36.68

p=0.0287 (Statistically significant p<0.05)

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

MALES CASES (33)		
PARAMETER	**CASES**	**FRQUENCY**
HYPERMETHYLATED	22	66.66% (22/33)
NON HYPERMETHYLATED	11	33.33% (11/33)

FEMALES CASES (17)		
PARAMETER	**CASES**	**FREQUENCY**
HYPERMETHYLATED	11	64.70% (11/17)
NON HYPERMETHYLATED	6	35.29% (6/17)

STATISTICAL ANALYSIS: Using chi square test

Odds ratio = 7.33, 95% C.I= 1.4-36.68,

$p = 0.08$ (Statistically insignificant $p > 0.05$)

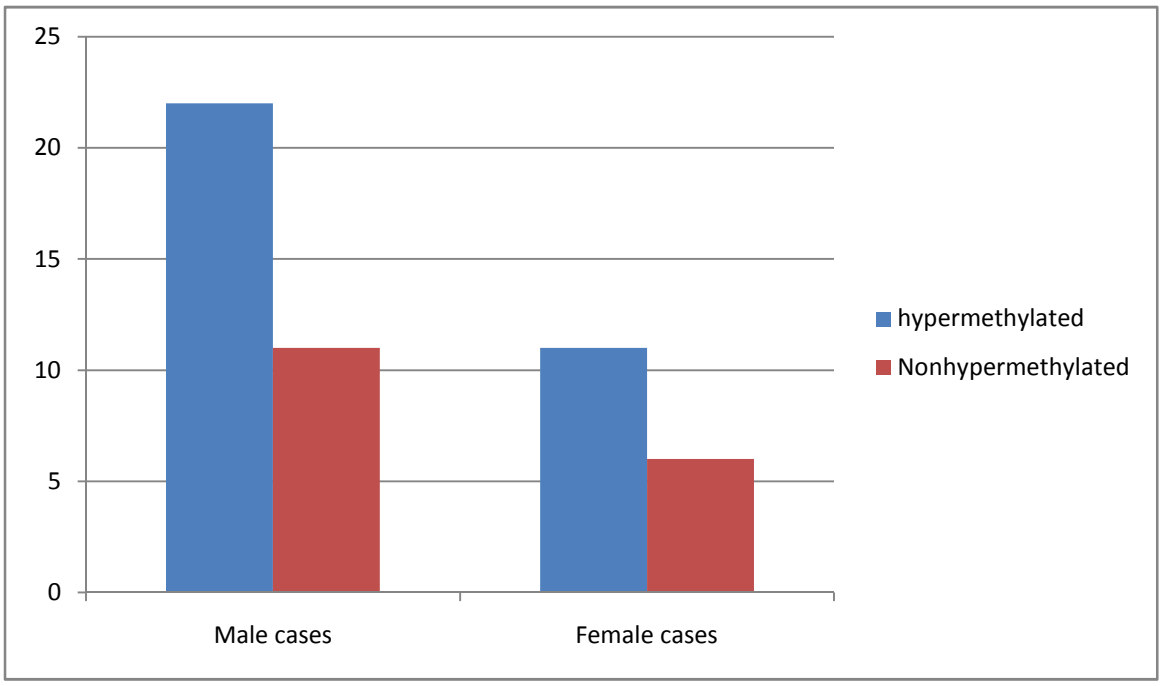

Fig 13: Histogram representing hypermethylated and nonhypermethylated male and female confirmed normal female cases.

O.D=0.916,

95% C.I=0.26-3.31

p=0.08 (Statistically insignificant p>0.05)

CHAPTER: 5

DISCUSSION

Gastric Cancer, the fourth most common cancer after lung cancer in the world, shows its high incidence in Japan, Iceland, China and other developing countries. Gastric cancer is an asymptomatic disease as it is not diagnosed in early stages which makes it the second most cause of cancer related deaths in the world. Gastric cancer is thought to result from a combination of environmental factors and the accumulation of generalized and specific genetic alterations, and consequently affects mainly older patients often after a long period of atrophic gastritis. Multiple genetic and epigenetic alterations are responsible for the development and progression of gastric cancer (Zheng *et al*., 2004), like activation of oncogenes, overexpression of growth factors/receptors, inactivation of tumor suppressor genes, DNA repair genes and cell adhesion molecules, and abnormalities of cell cycle regulators that define biological characteristics of cancer cells. Kashmir has a greater gastric malignancy rate with the frequency of 50-60 cases per 100000 persons & 63% of these occur in southern district of Kashmir.

Epigenetic mechanisms of gene inactivation, including promoter hypermemethylation, are undoubtedly important in cancer development and represent an alternative means of inactivating genes by transcriptional silencing mechanism. Epigenetics is a growing field of research. Transcriptional silencing by CpG island hypermethylation affects genes involved in all aspects of cell function and now rivals genetic changes that affect coding sequence as a critical trigger for neoplastic development and progression (Jones and Laird, 1999; Baylin and Herman, 2000). Gene promoter hypermethylation has become a target for developing strategies to provide molecular screening for early detection, diagnosis, prevention, treatment, and prognosis of cancer. The effectiveness of gene promoter hypermethylation for cancer screening and diagnosis ideally requires genes whose dysfunction early in tumor development, are specific to a particular cancer, and a biological fluid or access to tissue that is specific to the disease being assessed. For the majority of cancers, it is difficult to meet of three of these criteria. This approach involves the detection of gene promoter regions that are aberrantly hypermethylated in human tumors. This change is associated with an epigenetically mediated gene silencing that constitutes an alternative to coding region mutations for loss of gene function (Jones and Baylin, 2002; Herman and Baylin, 2003).

Nevertheless, the standard of proof for establishing that hypermethylation of the promoter of any given gene has a critical role in loss of gene expression and cancer development should probably be set quite high, regardless of whether the gene is a well established tumor suppressor gene, cell cycle regulatory gene, etc. So, in order to confirm this fact, it is worth evaluating the data a bit more critically.

The current study was thus aimed at understanding

- The promoter hypermethylation status of *TCF4* gene of Gastric cancer subjects of Kashmiri origin and their correlation with histopathologically confirmed controls.

DNA methylation involves addition of a methyl group to the carbon 5 position of the cytosine ring, catalyzed by DNA methyltransferases using S-adenosylmethionine as the donor molecule. The chromatin structure is modified during gene silencing by affecting acetylation, phosphorylation, methylation, ubiquitylation of histone tails (Jones and Baylin, 2002 ; Kelly *et al*., 2002). The rapid advance in the study of gene promoter hypermethylation in cancer was facilitated by the development of the Methylation Specific PCR (MSP) assay that allows for rapid detection of methylation in genes through the selective amplification of methylated alleles within a specific gene promoter (Herman *et al*., 1996). In the present study MSP was used for analysis of the methylation status of *TCF4* gene.MSP is much more sensitive than southern analysis, facilitating the detection of low members of methylated alleles and the study of DNA from small samples. Fresh human tumor samples often contain normal and tumor tissue, making the detection of changes specific for the tumor difficult. However, the sensitivity of MSP suggests that it would be useful for primary tumors as well, allowing for detection of aberrantly methylated alleles even if they contribute relatively little to the overall DNA in a sample.

In the present study, 50 histopathologically confirmed cancer cases and 30 histopathologically confirmed normal cases as controls were analyzed. Out of 50 cases, 33 were males which corresponded to 66% and hence remaining 17 were females corresponded to 34%. Hence Males: Females ratio was 1.94. However, among normal cases 15 were males and 15 were females with male to female ratio of 1:1. All the patients were symptomatic at the time of diagnosis.

The relationship between the promoter hypermethylation of *TCF4* gene with gender was observed. Occurrence of *TCF4* methylation was found to be unequally distributed in males and females with more frequency in males than in females. Among 33 males, 22 cases were found to be hypermethylated and 11 cases were unmethylated. In 33 methylated samples 5 samples were amplified with both sets of primers (methylated as well as unmethylated ones). This can be explained by the fact that excised tissue sample might be containing some unaffected tissue beyond cancer affected zone. However among 15 male controls 3 cases were hypermethylated and 12 were unmethylated. And among 17 female controls 11 cases were hypermethylated and 6 cases were unmethylated. The association of promoter hypermethylation with gastric cancer was found to be significant in males ($p= 0.0025$) as well as in females too ($p= 0.0028$). Also on comparing the male cases with female cases, 22 cases were found to be hypermethylated and 12 cases were unmethylated. The occurrence of *TCF4* methylation was found to be unequally distributed in males and females with more frequency in males than in females but the difference was not statistically significant ($p= 0.08$). The controls in both males and females show hypermethylation in some of the samples, this might be attributed to the contamination of non neoplastic cells with cancerous tissue or it might also predict precancerous lesions occurring in these patients or may also predict indication of metastasis. The low prevelance of gastric cancer in females than males may be contributed to high estrogen levels in females as it was observed that male rats are more prone to develop gastric cancer than females, but tumor numbers become similar after male rats are treated with estrogen (Furukawa *et al*., 1982)

The study also indicated high degree of association between gastric cancer and promoter hypermethylation of *TCF4* as study showed that more than 50% tissues were found to be expressed methylated *TCF4* promoter. Therefore, it is quite possible that like other geographical regions, methylation of promoter of *TCF4* gene is might be the major epigenetic event in gastric cancer in the Kashmir valley.

The evidence of methylation in control suggests its role in diagnosis. Premalignant detection of hypermethylation in gastric cancer patients have highlighted the potential importance of *TCF4* gene in early diagnosis as it was observed that promoter hypermethylation of *TCF4* gene was high in Stage I/II of patients than patients having disease in advanced age (Kim *et al*., 2008).So, detection of methylation

might represent the stage at which cancer may be in its course of development. Occurrence of methylation in individuals without diagnosed cancer might indicate that the patients may be in precancerous stage and so without further delay treatment can be made. Thus the current and previous studies lead us to conclude that mass evaluation of methylation status of this important gene could help and even prevent cancers well before they can be symptomized and diagnosed.

The rapeutic strategies targeting promoter hypermethylation may be highly beneficial in the Kashmiri population and other specific regions where incidence of gastric cancer is associated with high frequency of *TCF4* promoter hypermethylation. In summary, this is the first observational study to examine the status of promoter hypermethylation of *TCF4* gene in gastric 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.

The data thus gives a clue that *TCF4* gene expression can be readily and fully restored and growth rate of cancer cells decreased by treatment of cancer cells with demethylating agents and DNA methylation inhibitors. The administration of drugs such as cytosine analogs might be able to restore the function of *TCF4* gene and slow down the rate of gastric cancer progression. It also demonstrates that hypermethylation of *TCF4* gene can be designated as epigenetic biomarker for screening, diagnosis and prognosis of gastric cancer.

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APPENDIX

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

100bp DNA ladder	FERMENTAS / BIO ENZYME

PCR REAGENTS

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

DNA Isolation:

DNA was 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™ 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)

100bp DNA ladder (0.5µg/µl)