Cancer in Kashmir is reported as 50% according to statistics obtained from a period of 5 years (18.3%). In women, breast cancer (16.6%) is the leading one with an average frequency of 19.2%. It is more common in men than in women. Gastric adenocarcinoma accounts for 95% of malignant tumors of the stomach. The main cause appears to be a combination of environmental, dietary and genetic factors. The TCF4 gene is located at the 18q21.1 locus and is frequently inactivated by promoter methylation in a broad range of human tumors. The gene belongs to bHLH genes and is involved in the development and functioning of many different cell types. The aim of the study was to analyse the methylation status of CpG islands of TCF4 gene in primary and advanced stages of gastric cancer samples. A total of 100 gastric cancer patients histopathologically confirmed were analyzed from March 2011 to September 2012, among which 40 cases were in their primary stage and 60 in advanced stages. Hypermethylation analysis was done by using MSP after Bisulfite treatment of samples. The whole study was carried at Department of Biochemistry, Government Medical College, Srinagar. Hypermethylation level of TCF4 was significantly higher in early gastric type compared with advanced gastric tumors (P=0.003). The results suggest that inactivation of TCF4 by promoter methylation may play a role in the early stage of gastric carcinoma progression. The hypermethylation of TCF4 could be one reason for driving cell division uninterrupted. These results suggest that TCF4 gene will act as an biomarker for early gastric cancer detection.

**Keywords:** Gastric cancer; TCF4 gene, Hypermethylation, MSP (Methylation Specific PCR).

### INTRODUCTION

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.\(^1\) About 95% of stomach cancers are of adenocarcinoma type 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, Chile, and Iceland,\(^2\) although it is uncommon in the United States. It is more prevalent in males than females.\(^3\) Stomach cancer tends 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. Kashmir is a very high risk area of most common occurring cancers particularly cancers of gastrointestinal tract which comprise more than half the frequency of all the cancers. 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). The annual incidence of gastric cancer in Kashmir is reported as 50-60 per 100,000 individuals. 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.\(^4\)

Cancer can arise due to cumulative effect of mutations in various regulatory genes, or from epigenetic changes in DNA.\(^5,6,7\) 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. Epigenetics plays an important role in viral infections,\(^8\) cancer biology,\(^9,10\) activity of mobile elements,\(^11\) somatic gene therapy, cloning, transgenic technologies, genomic imprinting, developmental abnormalities, mental health, and X-inactivation.\(^12,13\) The power and promise of DNA methylation markers in early detection of cancer has been exciting as recent years have seen an explosion of interest in the epigenetics of cancer.

DNA methylation as one of the common epigenetic change, is a covalent chemical modification, resulting in the addition of a methyl (CH3) group at the carbon 5 position of the cytosine ring. The human genome contains regions of unmethylated segments interspersed by methylated ones.\(^14\) Approximately half of all the genes in humans have CpG islands.\(^9,11\) DNA methylation is brought about by a group of enzymes known as the DNA methyltransferases (DNMT’s), where methyl group is donated by SAM(S-adenosyl methyl transferases). As compared with normal cells, the malignant cells show major disruptions in their DNA methylation patterns.\(^15\) Many tumors show some kind of hypermethylation or even hypomethylation of one or more genes. Hypermethylation results in loss of expression of a variety of genes critical in the development of cancer by causing epigenetic silencing. This silencing is caused by either blocking transcription factors like AP-2, c-Myc/Myn, the
cyclic AMP-dependent activator CREB, E2F, and NFkB to promoter regions\textsuperscript{7,18} or by allowing repressors to bind promoter region of DNA like MBD1, MBD2, MeCP2, and Kaiso\textsuperscript{19}.

Hypermethylation is associated with many leukemias and other hematologic diseases. Many genes, such as the calcitonin gene,\textsuperscript{16}p15INK4B, p21Cip1/Waf1, the \textit{ER} gene, p16, Rasf1A, SDC4, MDR, and so on, were seen to be hypermethylated in a variety of hematologic cancers. On the basis of this criteria, We selected TCF4 gene, which is located on Chromosome no 18q21.1. TCF4 is a Wnt signaling pathway component- a pathway important in carcinogenesis \textsuperscript{20,21}. Deregulation of TCF4 is found in various cancer cell lines like colon, stomach etc. 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 highest levels are present in fetal brain. So its mutation can cause pit Hopkins syndrome-a neurodevelopmental disease. 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, which binds to E-boxes (CANNTG), found in the promoters of various important genes. 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\textsuperscript{22}.

**MATERIALS AND METHODS**

The study was a hospital based case-control study was undertaken to shed some light on the etiology of gastric cancer in Kashmir: 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. The gastric case and control tissue samples were collected from Department of Surgery S.M.H.S (Shri Maharaja Hari Singh) Hospital associated with Government Medical College, Srinagar. The study sample size was 100 out of which 40 cases were in stage I/II and 60 controls were in stage III/IV. All the samples were histopathologically confirmed before further processing. Record was maintained of complete case history of patients

**Collection of Tissue Samples**

The case and control samples obtained from Department of Surgery, S.M.H.S. Hospital, and private administered hospitals were put in plastic vials (50 ml volume) containing 10 ml of normal saline and DNA was extracted by kit based method (Quick-g DNA\textsuperscript{TM} Mini Prep) supplied by ZYMO RESEARCH.

**DNA Modification (Bisulfite Treatment)**

The above extracted Genomic DNA was modified by EZ DNA Methylation-Direct\textsuperscript{TM} Kit supplied by ZYMO RESEARCH.

The modified DNA contains uracil at all places where there were unmethylated cytosines before bisulphite treatment. 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\textsuperscript{23}.

**Methyl Specific Polymerase Chain Reaction (MSP)**

The principle of this PCR method lies in the amplification of the hypermethylated and non-methylated DNA of the same gene by different primer sequence; one for hypermethylated version of the gene and one for the non-methylated version of the same gene. Thus by visualizing 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 DNA sample was amplified using the following primer pairs, two for each gene\textsuperscript{24}.

<table>
<thead>
<tr>
<th>Nature of Sequence</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmethylated primer</td>
<td>Forward primer: 5’- TGA ATT TGT STT GTG TGT TTT T G - 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5’- AAA AAA AAC TCT CCA TAC ACC ACC - 3’</td>
</tr>
<tr>
<td>Methylated primer</td>
<td>Forward primer: 5’- GAA TTT GTA ATT TCG TGC GTT TC - 3’</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5’- AAA AAA AAC TCT CCG TAC ACC G - 3’</td>
</tr>
</tbody>
</table>

The amplified DNA were of approx same base pairs in length, the methylated band of 258 bp and the unmethylated band were of 259bp and were then visualized under UV light in presence of a 50/100 bp DNA ladder run parallel to the amplified PCR products on 2% ethidium bromide pre-loaded agarose gel.

**RESULTS**

In the present study 100 histopathologically confirmed gastric cancer cases belonging to Kashmir division were analyzed for promoter region hypermethylation of TCF4 gene. 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. So we have 60 gastric cancer samples in stage III/IV and 40 cases in stage I/II as shown in figure 1 and 2. Figure 3 and 4 shows representative gel picture of products of cases amplified by methylated and unmethylated primers.

**Analysis of TCF4 gene promoter hypermethylation in cases**

To determine the status of TCF4 promoter hypermethylation in gastric cancer cases from Kashmir
valley, we performed Methylation Specific PCR (MSP) for the promoter region (exon 1) of TCF4 gene in 100 surgically resected gastric cancer DNA Primers described were used to discriminate between methylated and unmethylated DNA following bisulfite treatment and to discriminate between DNA modified by bisulfite and that which had not been modified. The amplicons were analysed on 2% agarose gel. Amplification was carried out using hot start PCR method; the method involves heating the PCR mixture without using Taq polymerase up to 95°C for 5 min. and then adding Taq polymerase to it. This decreases the non specific amplifications. The PCR products of methylated and unmethylated bands were 258 and 259 bp respectively. Table 1 and 2 shows histogram of samples in different stages.

Figure 1: 76% (76/100) of the gastric cancer tissues shown methylated TCF4 promoter and 24% (24/100) of the cases showed unmethylated TCF4 promoter.

Figure 2: Histogram representing hypermethylated and non hypermethylated cases of gastric cancer in stage I/II with gastric cancer cases in stage III/IV.

Figure 3: Representing MSP (Methylation Specific PCR) Of Gastric cancer DNA samples run on 2% agarose gel. Product sizes: TCF4 Methylated, 258 bp: Ladder; 100 bp; M-Represents methylated product.

Bands in this figure shows methylated bands of gastric cancer samples (stage I/II) amplified by methylated primers only. However lane 3 and lane 7 do not show any bands.

Figure 4: Representing MSP (Methylation Specific PCR) Of Gastric cancer DNA samples (Stage III/IV) run on 2% agarose gel. Product sizes: TCF4 Methylated, 258 bp: Ladder; 100 bp; M-Represents methylated product.

Bands in this figure shows methylated bands of gastric cancer samples (stage III/IV) amplified by methylated primers only.

Thus, on comparing hypermethylation between early stage with advanced stage patients by Fischer exact test, the association of promoter hypermethylation with gastric cancer (p=0.03) and was thus found to be significant.

Table 1: Data representing no. of cases in stage I/II showing promoter hypermethylation and non-hypemethylation during MSP amplification in gastric cancer cases confirmed by 2% agarose gel electrophoresis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cases (N=40)</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypermethylated</td>
<td>35</td>
<td>87.5% (35/40)</td>
</tr>
<tr>
<td>Nonhypermethylated</td>
<td>5</td>
<td>12.5% (5/40)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cases (N=60)</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypermethylated</td>
<td>41</td>
<td>68.3% (41/60)</td>
</tr>
<tr>
<td>Nonhypermethylated</td>
<td>19</td>
<td>31.66% (19/60)</td>
</tr>
</tbody>
</table>
DISCUSSION

Gastric cancer is the most deadly disease especially in developing countries. It is a commonly diagnosed cancer in both men and women but more prevalent in males than females. It is diagnosed in advanced stage as it is an asymptomatic disease. In Kashmir valley this disease is highly prevalent due to ethnic background and different dietary habits. Recent progresses made in the field of molecular biology have shed light on the different alternative pathways involved in the gastric carcinogenesis, and more importantly cross talk among these pathways.25,26

DNA methylation as one of the epigenetic changes and involves addition of a methyl group to the carbon 5 position of the cytosine ring. This reaction is catalyzed by DNA methyltransferases in the context of the sequence 5’-CG-3’, which is also referred to as a CpG dinucleotide.27,28 Transcriptional silencing by CpG island hypermethylation affects genes involved in all aspects of normal cell function and now rivals genetic changes that affect coding sequence as a critical trigger for neoplastic development and progression27,28. 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.29

Gene promoter hypermethylation has become a target for developing strategies to provide molecular screening for early detection, diagnosis, prevention, treatment, and prognosis of cancer.

Occurrence of TCF4 methylation was found to be unequally distributed among patients in stage I/II than Stage III/IV, with more frequency in early stage patients than patients presenting disease in advanced age. Among 40 early stage patients, 35 cases were hypermethylated and 5 were unhypermethylated and among 60 advanced stage patients, 41 cases were hypermethylated and 19 were unhypermethylated. The association of promoter hypermethylation with gastric cancer (p=0.03) and is thus found to be significant.

Thus from our observations we observed that we get more number of cases in stage II/IV than in stage I/II. It can be due to its asymptomatic nature as stomach cancer is diagnosed very late when it spreads to lymph nodes. We received 60 patients in advanced stage in SMHS (Shri Maharaja Hari Singh) hospital in a period of 2 years. And only 40 patients in early stage gastric cancer. Our study observed more hypermethylation in early stage gastric cancer patients (87.5%) than in advanced stage gastric cancer patients (68.33%). So, it can be predicted that TCF4 gene shows hypermethylation early than other genes in cancer which shows promoter hypermethylation increases with advanced stages.

CONCLUSION

Our study concluded that TCF4 can be used as an early biomarker for gastric cancer diagnosis as this gene is an early gene which shows hypermethylation. So, TCF4 gene can be used to predict onset of gastric cancer so that early treatment can be made for decreasing survival of the disease. Besides prognosis can also be made after treatment of the disease.

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