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Distribution of P16 Promoter Hypermethylation in Male/Female Colorectal Cancer Patients of Kashmir Valley

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ABSTRACT: Colorectal cancer (CRC) being the commonest cancer is the major cause of mortality and morbidity worldwide. It is commonly known as bowel cancer and is the third most common cause of cancer-related deaths in the western world. Colorectal cancer has been reported to show geographical variation in its incidence, even within areas of ethnic homogeneity. Colorectal cancer development and progression is dictated by series of alterations in genes such as tumor suppressor genes, DNA repair genes, oncogenes and others. The aim of this study was to analyse the distribution of promoter hypermethylation of CpG islands of *p16* gene in male/female colorectal cancer patients of Kashmiri origin. The study was a case-control study. DNA was extracted from all the samples and was modified using bisulphite modification kit. Methylation-specific polymerase chain reaction was used for the analysis of the promoter hypermethylation status of *p16* gene. The epigenetic analysis revealed different *p16* promoter hypermethylation profile in male and female colorectal cancer patients of Kashmir Valley. However, in both cases association of promoter hypermethylation with colorectal cancer was found to be significant. Occurrence of *p16* promoter hypermethylation was found to be unequally distributed in males and females with more frequency in males than in females but the difference was not statistically significant(P =0.7635).

Keywords: Colorectal cancer, Promoter, Hypermethylation, Kashmir Valley, Tumor suppressor gene, *p16* gene

I. INTRODUCTION

Colorectal cancer (CRC) being the commonest cancer is the major cause of mortality and morbidity worldwide. CRC commonly known as colon cancer or large bowel cancer includes cancerous growths in the colon, rectum and appendix. It is a commonly diagnosed cancer in both men and women. There are 6,55,000 deaths worldwide per year and it is the fifth most common form of cancer in the United States and the third leading cause of cancer-related deaths in the Western world (1,2). The incidence of this malignancy shows considerable variation among racially or ethnically defined populations in multiracial/ethnic countries. Kashmir has been reported as a high-incidence area of GIT cancers (3, 4). Colorectal Cancer in Kashmir valley is the third most common GIT cancer after esophageal and gastric cancer (5). The development of CRC is a multistep process, which can arise due to the cumulative effect of mutations in various different proto-oncogenes, tumor suppressor genes, and/or from epigenetic changes in DNA (6, 7). DNA methylation has been shown to play a central role in gene imprinting, embryonic development, chromosome gene silencing, and cell cycle regulation. The majority of DNA methylation in mammals occurs in 5'-CpG-3' dinucleotides, but other methylation patterns do exist. In fact, about 80 percent of all 5'-CpG-3' dinucleotides in mammalian genomes are found to be methylated, whereas the majority of the 20 percent that remain unmethylated are within promoters or in the first exons of genes. Aberrant methylation of normally unmethylated CpG islands has been associated with transcriptional inactivation of gene (8). In many disease processes such as cancer, gene promoter CpG islands acquire abnormal hypermethylation, which results in transcriptional silencing (9). Promoter hypermethylation can begin very early in tumor progression and is one of the hallmarks of carcinogenesis associated with transcriptional silencing and loss of expression of genes encoding for diverse cellular pathways (10). The *p16* (*CDKN2a*/*INK4a*) gene is an important tumor-suppressor gene located on human chromosome 9 in the region 9p21 and is involved in the *p16*/cyclin dependent kinase/retinoblastoma gene pathway of cell cycle control, in which the *p16* protein is considered to be a negative regulator involved in the inhibition of G1 phase progression (11). The human *p16* protein is a 16-KD protein containing 156 amino acids and was first discovered in a yeast two-hybrid system to detect proteins that interact with human cyclin dependent kinase

(12). Increased expression of the *p16* gene as organisms age reduces the proliferation of stem cells (13). This reduction in the division and production of stem cells protects against cancer while increasing the risks associated with senescence. Human *p16* gene possesses a CpG island in the promoter region and it has been reported that the methylation of discrete regions of the *p16* CpG island is associated with the silencing of the gene. Promoter hypermethylation of *p16* gene in colorectal cancer patients has been studied and documented in several studies. Some studies have suggested that *p16* plays an important role in cancer pathogenesis and has implications for improving the clinical management (14). Methylation of the *p16* (*INK4a*) gene has been shown to contribute to the process of carcinogenesis in colorectal cancer and is useful as a prognostic factor in the early stage (15, 16). *P16* might act as a tumor suppressor in colorectal carcinomas and has been shown to be frequently methylated in advanced colorectal carcinomas (17, 18). *P16* hypermethylation has been documented to play a role in the carcinogenesis of colorectal cancers (19). Epigenetic mechanisms of gene inactivation, including promoter hypermethylation, are undoubtedly important in cancer development and represent an alternative means of inactivating tumor suppressor genes. The present work was an attempt to study the association and distribution of *p16* gene promoter hypermethylation in male and female colorectal carcinoma patients of Kashmir valley as this aspect has not been well studied.

II. MATERIALS AND METHODS

COLLECTION OF TISSUE SAMPLES

The study included 70 surgically obtained colorectal samples among which 50 were cases and 20 were normal colorectal samples. The samples were obtained from the Department of Surgery, Shri Maharaja Hari Singh (S.M.H.S) hospital an associated hospital of Government Medical College Srinagar and were put in sterilized plastic vials (50 ml volume) containing 10 ml of normal saline and transported from the theatres to the laboratory on ice and stored at -80 ^{o} C for further analysis. Also, a part of each sample was sent to histopathology laboratory of S.M.H.S hospital for histopathological confirmation. The information regarding the gender for each sample was collected from the histopathological reports. All ethical considerations were taken care of during the study and the recruitment process was started only after ethical clearance from the Departmental Ethical Committee as per norms.

III. GENETIC ANALYSIS

Extraction of genomic DNA

For the isolation of genomic DNA, kit based method was used. The kit used was Quick- g DNA^{TM} MiniPrep supplied by ZYMO RESEARCH. The protocol followed was as per kit. The DNA eluted was stored at 4^{0}C for a short time and then the vials were kept at -20^{o}C for longer duration of time.

QUALITATIVE AND QUANTITATIVE ANALYSIS OF GENOMIC DNA

The integrity of the genomic DNA was examined by gel electrophoresis using 1 % agarose gel. The quantity of the DNA was determined by measuring optical density at 260nm and 280 nm by double beam spectrophotometer (Evolution 60S from Thermo Scientific). The ratio of 260/280nm was calculated and the DNA samples for which the ratio was 1.7-1.9 were considered for the future use. DNA was alliqouted into three to four tubes so as to protect damage from freeze thawing and stored in -20^{o}C freezer for longer duration of time.

DNA MODIFICATION (BISULFITE TREATMENT)

DNA modification **(**i, e., sodium bisulfite treatment) converted unmethylated cytosines to uracil and hence enabled to distinguish between the hypermethylated and non hypermethylated cytosine residues. DNA was modified by kit based method and the kit used was EZ DNA Methylation^{TM} Kit supplied by ZYMO RESEARCH. The modified DNA was stored at -20^{0}C for further use.

METHYL SPECFIC POLYMERASE CHAIN REACTION (MSP) Amplification of the promoter region of the *p16* gene was carried out in Eppendorf Gradient Thermalcycler in a 25µl reaction mixture as shown in table I. Reactions were hot-started at 95^{o}C for 5 min. The primers used and thermal cycling conditions are given in Table II and III. Controls without DNA were performed for each set of PCR. Universal Methylated Human DNA Standard and Control with primers was used as positive control, and Lymphocyte DNA was used as negative control.

Table I: Volume and concentrations of different reagents used in MSP

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

Table II: Primers described by Herman (20) used and length of fragments obtained in MSP

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

Table III: Thermal cycling conditions

Note: The annealing temperature for unmethylated and methylated *p16* reaction was 60^0C and 65^0C respectively.

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

IV. STATISTICAL ANALYSIS

The Fishers exact test was used in studying the male and female groups. Odds ratios 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.

V. RESULTS

Methylation-specific PCR was done to examine the methylation status of the promoter region of *p16* gene. The relationship between the promoter hypermethylation of *p16* gene and selected clinicopathological parameter, gender was examined. Among 29 males, 20 cases were hypermethylated and 9 cases were unhypermethylated and among 10 male controls, 2 cases were hypermethylated and 8 cases were unhypermethylated (Fig. 1). As shown in gel picture among the methylated cases few cases also showed the presence of unmethylated DNA that could be derived from unmethylated DNA of normal, adjoining mucosal

cells and tumor cells as well as normal constituents in the stroma such as vascular endothelial cells, smooth muscles, fibroblasts and inflammatory cells. The association of promoter hypermethylation with colorectal cancer was evaluated using Fisher's exact test and was found to be significant in males (P =0.0107, Odds ratio=8.889 and 95% C.I=1.563 to 50.55). In comparison, among 21 females, 13 cases were hypermethylated and 8 cases were unhypermethylated and among 10 female controls 1 case was hypermethylated and 9 cases were unhypermethylated (Fig.2). The association of promoter hypermethylation with colorectal cancer was again evaluated using Fisher's exact test and was found to be significant in females too (P =0.0089,Odds ratio=14.63 and 95% C.I=1.547 to 138.3).

However, on comparing the male cases with female cases, 20 cases were hypermethylated and 9 cases were unhypermethylated in males and 13 cases were hypermethylated and 8 cases were unhypermethylated in females, occurrence of *p16* methylation was found to be unequally distributed in males and females with more frequency in males than in females but the difference was not statistically significant (*P* =0.7635, Odds ratio=1.368 and 95% C.I=0.4197 to 4.456).

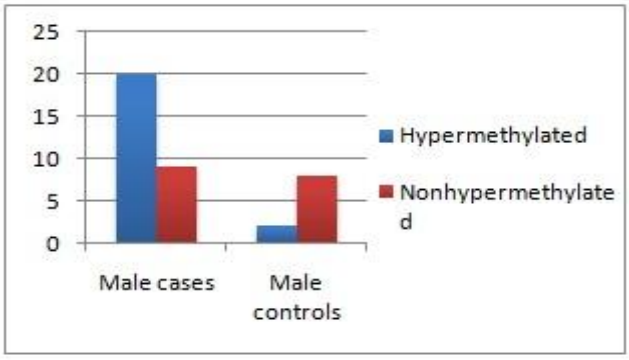

Fig.1: Histogram representing hypermethylated and nonhypermethylated male colorectal cancer cases and histopathologically confirmed normal male cases

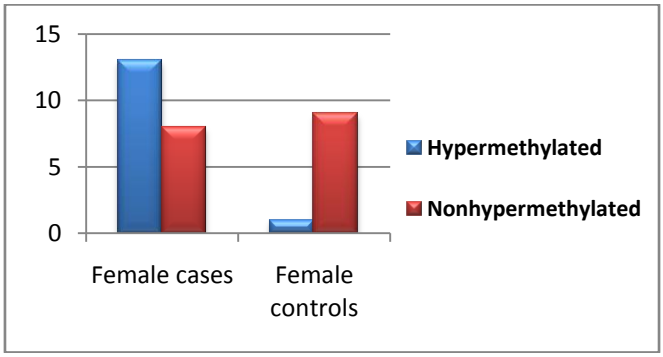

Fig.2: Histogram representing hypermethylated and nonhypermethylated female colorectal cancer cases and histopathologically confirmed normal female cases

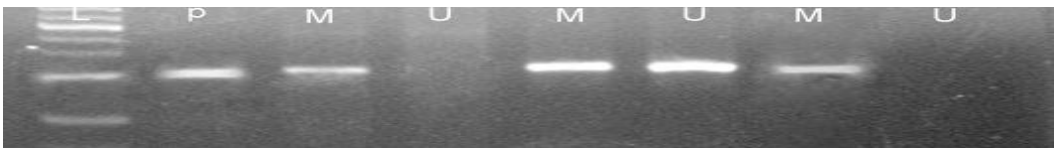

Fig. 3. Representing MSP (Methylation Specific PCR) of some male colorectal cancer DNA samples run on 2% agarose gel.

Lane 1- Represents 50 bp ladder
Lane 2-Represents positive control (universal methylated DNA) amplified with methylated primer
Lane 3 and 4- Represents case 1 amplified with only methylated primer
Lane 5 and 6- Represents case 3 amplified with both primers
Lane 7 and 8- Represents case8 amplified with only methylated primer

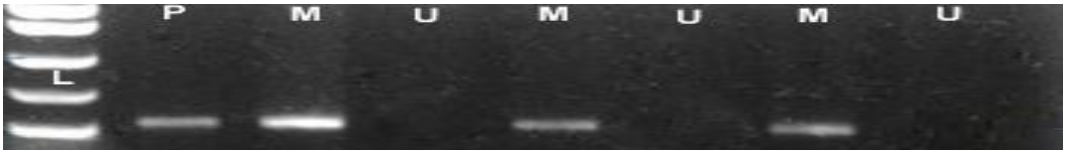

Fig. 4. Representing MSP (Methylation Specific PCR) of some female colorectal cancer DNA samples run on 2% agarose gel.

Lane 1- Represents 50 bp ladder
Lane 2-Represents positive control (universal methylated DNA) amplified with methylated primer
Lane 3 and 4- Represents case 2 amplified with only methylated primer
Lane 5 and 6- Represents case 5 amplified with only unmethylated primer
Lane 7 and 8- Represents case 15 amplified with only methylated primer

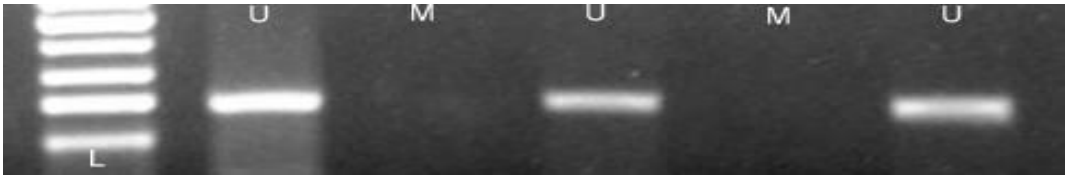

Fig. 5. Representing MSP (Methylation Specific PCR) of histopathologically confirmed normal colorectal DNA samples run on 2% agarose gel.

Lane 1- Represents 50 bp ladder
Lane 2-Represents negative control (lymphocyte DNA) amplified with unmethylated primer
Lane 3 and 4- Represents normal male case 1 amplified with only unmethylated primer
Lane 5 and 6- Represents normal female case 7 amplified with only unmethylated primer

VI. DISCUSSION

Colorectal cancer is one of the most aggressive malignancies and occurs at a high incidence in most countries with many deaths (21). It is one of the commonly diagnosed cancer in both men and women. Most colorectal cancers (CRC) develop through multiple mutations in the normal colonic mucosa, and evolve through the adenoma-carcinoma sequence (7, 22). Recent progresses made in the field of molecular biology have shed light on the different alternative pathways involved in the colorectal carcinogenesis, and more importantly cross talk among these pathways (23, 24). Tumor suppressor genes were initially hypothesized to be inactivated in cancer cells as a result of genetic defects of both alleles (i.e., the Knudson two-hit hypothesis). However, there is now evidence that epigenetic events, such as hypermethylation of cytosine–guanine (CpG) sites in regulatory regions (e.g., the promoter), may be a critical alternative mechanism of tumor suppressor gene inactivation. DNA methylation involves addition of a methyl group to the carbon 5 position of the cytosine ring catalyzed by DNA methyltransferases in the context of the sequence 5'-CG-3', which is also referred to as a CpG dinucleotide (25, 26). When methylation occurs within a CpG island located in the promoter region of a gene, it is accompanied by changes in chromatin composition around the island that denies access to regulatory proteins needed for transcription. Transcriptional silencing by CpG island hypermethylation affects genes involved in all aspects of normal cell function and now rivals genetic changes that affect coding sequence as a critical trigger for neoplastic development and progression (26, 27). 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 (28). Gene promoter hypermethylation has become a target for developing strategies to provide molecular screening for early detection, diagnosis, prevention, treatment, and prognosis of cancer. This approach involves the detection of gene promoter regions that are aberrantly hypermethylated in human tumours. This change is associated with an epigenetically mediated gene silencing that constitutes an alternative to coding region mutations for loss of gene function and involves the modification of both the genetic and histone code (29). *P16* tumour suppressor gene plays a monitor role in the passage of cells through the G1 to S phase of the cell cycle by binding to cyclin-dependent kinase 4 and inhibiting its effect on cyclin D1 (30-32). Mutations in the CDKN2A gene are associated with increased risk of a wide range of cancers and alterations of the gene are frequently seen in cancer cell lines. Human *p16* gene possesses a CpG island in the promoter region and it has been reported that the methylation of discrete regions of the *p16* CpG island is associated with the silencing of the gene. The exon 1 coding sequences of the *p16* gene resides within 5' CpG islands. This area is not methylated in most normal tissues but methylated in many human cancers. Methylation of cytosine residues at CpG sites in *p16* gene promotor, resulting in a silenced p16 expression, has been reported in many cell lines, including CRC, and some primary carcinomas in varied origins, such as colon, brain, breast, bladder, ovary, lung, and myeloma and so on (33). Promoter hypermethylation of *p16* gene in colorectal cancer patients has been studied and analyzed in several studies. Some studies have suggested that *p16* plays an

important role in cancer pathogenesis and has implications for improving the clinical management (14). Methylation of the *p16* (*INK4a*) gene has been documented to contribute to the process of carcinogenesis in colorectal cancer and is useful as a prognostic factor in the early stage (15, 16). *P16* might act as a tumor suppressor in colorectal carcinomas and is more frequently methylated in advanced colorectal carcinomas (18, 34). *P16* hypermethylation might play a role in the carcinogenesis of colorectal cancers (19). The inactivation of *p16INK4a* gene due to aberrant promoter hypermethylation in esophageal, lung, gastric and hepatocellular carcinoma has been well documented (4, 17, 35-39). Epigenetic mechanisms of gene inactivation, including promoter hypermethylation, are undoubtedly important in cancer development and represent an alternative means of inactivating tumor suppressor genes. Considering the important role of promoter hypermethylation in inactivation of *p16* which is one of the most frequently altered genes in squamous cell carcinoma of esophagus and many other human cancers, in the present study, the distribution of *p16* promoter hypermethylation was investigated in male and female colorectal carcinoma patients from Kashmir valley where frequency of colorectal cancer is higher. The male to female ratio of the cancer patients came to be 1.38. All the patients were symptomatic at the time of diagnosis. Clinicopathological data revealed that the patients presented with abdominal pain, change in bowel habits, rectal bleeding and loss of appetite. The other signs and symptoms were subjective weight loss, abdominal mass, vomiting or abdominal distention and anemia.

In the present study MSP was used for analysis of the methylation status of *p16* gene. This method provided significant advantages over previous ones used for assaying methylation. MSP is much more sensitive than Southern analysis, facilitating the detection of low numbers of methylated alleles and the study of DNA from small samples. MSP allows examination of all CpG sites, not just those within sequences recognized by methylation sensitive restriction enzymes. The genetic analysis revealed that unlike other high risk regions, Kashmiri population has a different hypermethylation profile of *p16* gene promoter in males and females. Though there was no selection bias in sampling, occurrence of *p16* methylation was found to be unequally distributed in males and females with more frequency in males than in females.

Therapeutic strategies targeting promoter hypermethylation may be highly beneficial in the Kashmiri population and other specific regions where incidence of colorectal cancer is associated with high frequency of *p16* promoter methylation. The data gives a clue that *p16* gene expression can be readily and fully restored and growth rate of cancer cells decreased by treatment of cancer cells with demethylating agents and DNA methylation inhibitors. The administration of drugs such as cytosine analogs might be able to restore the function of these tumour suppressor genes and slow the rate of colorectal cancer progression.

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