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ORIGINAL ARTICLE

Methylation Status of hMLH1 Gene in Colorectal Cancer Patients in Ethnic Population of Kashmir Valley

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A R T I C L E I N F O

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A B S T R A C T

Background: Colorectal cancer is **c**ommonly known as bowel cancer, its development and progression is dictated by chain of alterations in genes such as tumor suppressor genes, DNA repair genes, oncogenes and others.

Method: The present work was a case control study aiming to ascertain the role of promoter methylation of CpG islands of hMLH1 gene in colorectal cancer patients among the Kashmiri population. 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 methylation status of hMLH1 gene.

Results: The epigenetic analysis revealed that unlike other high risk regions, Kashmiri population has a different promoter methylation profile of hMLH1 gene as 67.5 % of the cases showed hMLH1 promoter methylation in comparison to 15 % of the normal cases which also showed promoter methylation of hMLH1 gene. The association of promoter methylation with colorectal cancer was found to be significant (P=0.0006). Occurrence of hMLH1 promoter 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). Similarly, frequency of hMLH1 promoter methylation was found to be certainly higher in Stage III/IV (85.71%) compared to Stage I/ II (57.69%) but the difference was not statistically significant (P=0.0673).

Conclusion: The results suggest that hMLH1 aberrant promoter methylation in Kashmiri population contributes to the process of carcinogenesis in colorectal cancer and is reportedly one of the commonest epigenetic changes in the development of colorectal cancer.

INTRODUCTION

Colorectal cancer is one of the most aggressive malignancies and occurs at a high incidence in most countries (1). It is a 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. Recent advancements 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 (2). Colon Cancer/Colorectal Cancer is one of the three leading causes of cancer mortality world-wide, with an inci-

dence of approximately 1 million cases and a mortality of 500,000 annually.

DNA methylation is one of the most commonly occurring epigenetic events taking place in the mammalian genome. This change, though heritable, is reversible, making it a therapeutic target. The human genome is not methylated uniformly and contains regions of unmethylated segments interspersed by methylated regions. In contrast to the rest of the genome, smaller regions of DNA, called CpG islands, ranging from 0.5 to 5 kb and occurring on average every 100 kb, have distinctive properties. These regions are unmethylated, GC rich (60% to 70%), have a ratio of CpG to GpC of at least 0.6, and thus do not show any suppression of the frequency of the dinucleotide CpG (3). Approximately half of all the genes in humans have CpG islands and these are present on both housekeeping genes and genes with tissue-specific patterns of expression.

DNA methylation is brought about by a group of enzymes known as the DNA methyltransferases (DNMT). The DNMTs known to date are DNMT1, DNMT1b, DNMT1o, DNMT1p, DNMT2, DNMT3A, DNMT3b with its isoforms, and DNMT3L (Robertson KD). Methylation can be de novo (when CpG dinucleotides on both DNA strands are unmethylated) or maintenance (when CpG dinucleotides on one strand are methylated). DNMT1 has de novo as well as maintenance methyltransferase activity, and DNMT3A and DNMT3b are powerful de novo methyltransferases (4). The importance of these enzymes has been shown using several mouse experiments in which the mouse deficient in the gene dies early in development or immediately after birth.

In addition to the DNMTs, the other machinery of methylation includes demethylases, methylation centers triggering DNA methylation, and methylation protection centers. DNA methylation patterns are established early in embryogenesis and are very finely controlled during development. The enzymes that actively demethylate DNA include 5-methylcytosine glycosylase, which removes the methylated cytosine from DNA, leaving the deoxyribose intact (5) (eventually local DNA repair adds back the cytosine in nucleotide form), and MBD2b, which refers to an isoform that results from initiation of translation at the second methionine codon of the gene encoding methyl-CpG binding domain 2 (MBD2) protein (6). MBD2b lacks glycosylase or nuclease activity and is thought to cause demethylation by hydrolyzing 5-methylcytosine to cytosine and methanol. However, two independent laboratories have not been able to reproduce these results in mammalian and *Xenopus* systems (7).

There are over 300 hMLH1 germline mutations described all along the gene that cause hereditary non-polyposis colorectal cancer (HNPCC). These mutations are not present in any particular hotspot or zone of the gene and include either nucleotide substitutions (missense, nonsense or splicing errors) or insertions/deletions (gross or small). In most of these mutations the resulting protein is truncated. There are also founding mutations which account for a high proportion of the HNPCC tumours in some specific populations (for example there are two Finnish mutations that delete the exons 16 or 6). Some germline genetic changes have also been described in both exons and introns as non pathogenic.

MATERIAL AND METHODS

Collection of Tissue Samples: The study included 60 surgically obtained colorectal samples among which 40 were CRC patients and 20 were normal colorectal samples. The carcinoma and control samples were obtained from the Department of Surgery, Sher-e-Kashmir Institute of Medical sciences (SKIMS) and Department of Surgery, of 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 operation theatres to the laboratory on ice and stored at -80 ^{o} C for further analysis. The information regarding the gender, stage and histological grade for each carcinoma sample was collected from the histopathological reports. The information regarding the gender of control samples was also collected.

Genetic Analysis

Extraction of genomic DNA: For the isolation of genomic DNA, phenol / chloroform protocol based method as described in "Sambrook-Russell method". The eluted DNA was stored at 4^{0} C for a short time and then the vials were kept -20^{0}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 260 nm and 280 nm by Double Beam Spectrophotometer (HITACHI-U-1800). 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 by 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 (EZ DNA Methylation ™ Kit) supplied by ZYMO RESEARCH. The modified DNA was stored at -20^{0}C for further use.

Methyl Specific Polymerase Chain Reaction (MSP)

Amplification of the promoter region of the *hMLH1* gene was carried out in Eppendorf's Gradient Thermal Cycler in a 25µl reaction mixture as shown in table I. Reactions were hot-started at 95°C for 5 min. The primers used and thermal cycling conditions are given in Table II and III.

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

Reagent	Concentration	Volume
PCR MM (master mix)		12.5 µl
Forward primer	10 pmol/µl	1µl
Reverse primer	10 pmol/µl	1 µl
DNA sample	250 ng/µl	2 µl
Deionised water		8.5 µl
Total volume		***25* µl**

Table II: Primers described by Herman (25) used and length of fragments obtained in MSP (Methylation Specific PCR)

Nature of Primer		Primer sequence	Size of Amplicon
Unmethylated Primer	Forward primer	5′TTTTGATGTAGATGTTTATTA3′	115 bp
	Reverse primer	5′ACCACCTCATCATAACTACCC3′	
Methylated Primer	Forward primer	5′ACGTAGACGTTTTATTAGGGTCGC3′	124bp
	Reverse primer	5′ACCACCTCATCATAACTACCCACA3′	

Statistical Analysis: Statistical comparisons were performed using the SPSS (stastical product & service solutions) software package. Associations were determined with the χ^2-test for examining the differences in the distribution of *hMLH1* gene promoter methylation and unmethylation between cases and controls and Fishers exact test was used in case of studying the male and female groups.

Table III: Thermal cycling conditions

Steps	Temp °C	Time	Number of cycles
1. Hot-Start	95	5 min	1
2. Denaturation	95	30 sec	
3. Annealing	60/60	30 sec	35
4. Extension	72	30 sec	
5. Final extension	72	5 min	1

Note: The annealing temperature for both unmethylated and methylated *hMLH1* reaction was 60°C.

RESULTS

Methylation Specific PCR was done to examine the methylation status of the promoter region of *hMLH1* gene. As shown in table IV 67.5% (27/40) of the colorectal cancer tissues showed methylated *hMLH1* promoter and 32.5% (13/40) of the cases however showed unmethylated *hMLH1* promoter. Almost all 85% (17/20) of the histopathologically confirmed normal tissues showed unmethylated *hMLH1* promoter except only in three cases where *hMLH1* promoter was found to be methylated. The association of promoter hypermethylation with colorectal cancer was evaluated by χ^2 (Chi square) test and was found to be significant (P=0.0006, Odds ratio=7.765, 95% C.I=2.242 to 26.90).

Relationship between promoter methylation of *hMLH1* gene and selected clinicopathological parameters

The relationship between the promoter methylation of *hMLH1* gene and selected clinicopathological parameters was examined. These parameters included gender & clinical staging.

Table IV: Representing methylated and unmethylated cases of colorectal cancer and histopathologically confirmed normal controls

	Methylated (n=30)	Unmethylated (n=30)	Total
Cases	27(67.50%)	13(32.50%)	**40**
Males	16	7	
Females	11	6	
Controls	3(15%)	17(85%)	**20**
Males	2	8	
Females	1	9	

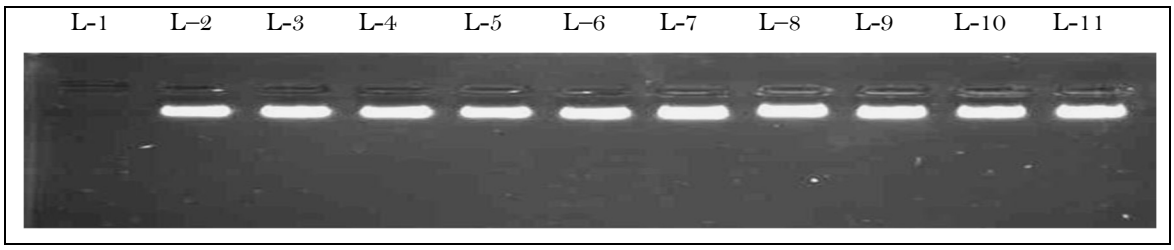
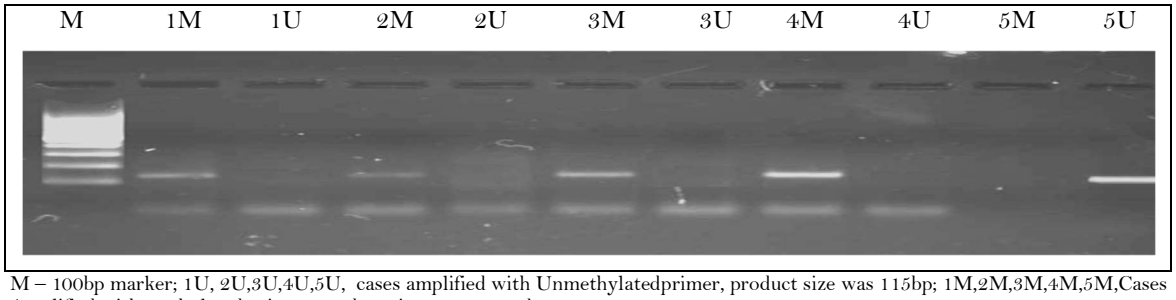


Figure II a–Lane (L) 2-9 showing the isolated DNA of case samples, run on 1% agarose gel



M – 100bp marker; 1U, 2U,3U,4U,5U, cases amplified with Unmethylatedprimer, product size was 115bp; 1M,2M,3M,4M,5M,Cases Amplified with methylated primer, product size was as 124 bp

Figure II b: MSP (Methylation specific PCR) of colorectal cancer DNA samples run of 2% agarose gel

Relationship of promoter methylation of *hMLH1* gene with colorectal cancer in males and females

Among 23 males, 16 cases were methylated and 7 cases were unmethylated and among 10 male controls, 2 cases were methylated and 8 cases were unmethylated. The association of promoter methylation 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 17 females, 11 cases were methylated and 6 cases were unmethylated and among 10 female controls 1 case was methylated and 9 cases were unmethylated. The association of promoter methylation 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, 16 cases were methylated and 7 cases were unmethylated in males and 11 cases were methylated and 6 cases were unmethylated in females. Occurrence of *hMLH1* methylation was found to be unequally distributed in males and females with more frequency in males than in females but the difference was not statistically significant (P =0.7635, Odds ratio=1.368 and 95% C.I=0.4197 to 4.456).

Table V: Representing no. of cases showing promoter methylation and unmethylation in stage I/II and stage III/IV during MSP amplification confirmed by 2% agarose gel electrophoresis

Stages	Methylated (n=27)	Unmethylated (n=13)	Total (n=40)
Stage I/II	15(57.69%)	11(42.30%)	26
Stage III /IV	12(85.71 %)	2(14.28 %)	14

Relationship of promoter methylation of *hMLH1* gene in stage I/ II and stage III/IV

There were 26 cases that were in Stage I and Stage II of the disease. Among these cases 15 cases were methylated and 11 cases were unmethylated. However, among 14 cases that were in Stage III and Stage IV of the disease, 12 cases were methylated and 2 cases were unmethylated (Table V). When the frequency of *hMLH1* promoter methylation was compared with clinical staging of the disease, *hMLH1* promoter methylation was found to be certainly higher in Stage III/IV (85.71%) compared to Stage I/ II (57.69%) but the difference was not statistically significant (P =0.0673, Odds ratio=3.889 and 95% C.I=0.9370 to 16.14) (Fisher's exact test).

DISCUSSION

Colorectal cancer is one of the most aggressive malignancies and occurs at a high incidence in most countries (8). It is a 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 (9, 10). 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 (11, 12). Various endogenous and exogenous agents from environmental exposures are constantly damaging DNA, and in combination with low DNA repair capacity this have been interpreted as increasing the likelihood of cancer development. Colon being the waste processor of our body is in particular exposed to a wide array of endo as well as exogenous chemicals. One of the most important treatments of this fatal cancer is surgery and subsequent chemotherapy and radiotherapy. For this purpose, it is important to identify the occurrence of genetic alterations as a new parameter to estimate the malignancy of the cancer.

Tumor suppressor genes were initially hypothesized to be inactivated in cancer cells as a result of genetic defects of both alleles (i.e., the Knudson two-hit hypothesis). However, there is now evidence that epigenetic events, such as hypermethylation of cytosine–guanine (CpG) sites in regulatory regions (e.g., the promoter), may be a critical alternative mechanism of tumor suppressor gene inactivation. 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 (13, 14). The methylation of gene, particularly the methylation of CpG-rich promoters,

could block transcriptional activation (13, 14). 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. The chromatin structure is modified during gene silencing by affecting acetylation, phosphorylation, methylation, and/or ubiquitylation of histone tails (15, 16). 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 (14, 17). 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 (18). 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 (15, 19) and involves the modification of both the genetic and histone code (15).

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 methylation 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 (20). The methylation of gene, particularly the methylation of CpG-rich promoters, could block transcriptional activation. 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. The chromatin structure is modified during gene silencing by affecting acetylation, phosphorylation, methylation, and/or ubiquitylation of histone tails. Transcriptional silencing by CpG island methylation 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. The rapid advance in the study of gene-promoter methylation 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. Gene promoter methylation 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 methylated 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 (21).

Considering the important role of promoter methylation in inactivation of *hMLH1 gene* which is one of the most frequently altered genes in gastric, endometrium and other human cancers. In the present study, the level of *hMLH1* promoter methylation was investigated in colorectal carcinoma tissues of patients from Kashmir valley where frequency of colorectal cancer is higher. The male to female ratio of the cancer came to be 1.5. 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 *hMLH1*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. In the present study, frequent promoter methylation of *hMLH1* gene was observed in colorectal cancer cases and methylation was significantly found to be associated with CRC. This result suggested that *hMLH1* methylation might link to a more malignant outcome of CRC. The genetic analysis of the cancer and normal cases revealed that unlike other high risk regions, Kashmiri population has a different methylation profile of *hMLH1* promoter. The study on colorectal cancer showed that more than 50% tissues expressed methylated *hMLH1* promoter. Therefore, it is quite possible that like other geographical regions, methylation of promoter of *hMLH1* gene is the major epigenetic event in colorectal cancer in the Kashmir valley. We observed completely methylated *hMLH1* promoter in 27 cancer cases out of 40. Though there was no selection bias in sampling, occurrence of *hMLH1* methylation was found to be unequally distributed in males and females with more frequency in males than in females. The hMLH1 promoter methylation was found to be certainly higher in Stage III/IV compared to Stage I/ II but the difference was not statistically significant.

This study demonstrates that *hMLH1* promoter methylation is a frequent epigenetic event in colorectal cancer of the Kashmir region. These results also indicate that *hMLH1* aberrant methylation may play an important role in colorectal cancer development. Therapeutic strategies targeting promoter hypermethylation may be highly beneficial in the Kashmiri population and other specific regions where incidence of colorectal cancer is associated with high frequency of *hMLH1* promoter methylation. The data gives a clue that *hMLH1* 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.

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