RESEARCH ARTICLE

Promoter methylation and immunohistochemical expression of hMLH1 and hMSH2 in sporadic colorectal cancer: a study from India

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Abstract To determine the etiological factors of human colorectal cancer (CRC) we assessed the frequency and prognostic significance of *hMLH1* and *hMSH2* genes in conjunction with hMLH1 and hMSH2 protein expression in 30 Indian CRC patients. The protein expression and promoter methylation of hMLH1 and hMSH2; Mismatch Repair genes (MMR) were analyzed by immunohistochemistry and methylation-specific PCR (MSP), respectively. A loss of hMLH1 expression was recognized in 4(13.3 %) and loss of hMSH2 expression was recognized in 2(6.6 %) of 30 CRC cases whereas 50 % tumors showed reduced expression of hMLH1 and 33.3 % showed reduced expression of hMSH2 protein. One tumor showed a loss of both hMLH1 and hMSH2 expression. Normal nuclear staining pattern of hMLH1 and hMSH2 was observed in almost all the adjoining and normal mucosa. Promoter hypermethylation of the *hMLH1* gene was detected in 15 of 30 CRC cases (50 %) and of *hMSH2* gene was only in 3 of 30 CRC cases (10 %). No promoter methylation of *hMLH1* and *hMSH2* genes was observed in adjoining and normal mucosa. Combination of methylation of *hMLH1* and *hMSH2* gene was observed in two tumors (6.6 %). A significant correlation between histological grade of the tumor, methylation and expression of *hMLH1* gene ($p < 0.05$) was observed. Normal expression of hMLH1 and hMSH2 was seen in all of the unmethylated tumors (100 %). Nuclear staining and promoter methylation of hMLH1 and hMSH2 did not significantly influence survival. *hMLH1* methylation was common and was significantly correlated with loss of hMLH1 protein expression. In contrast, *hMSH2* methylation was infrequent. These findings suggest that the inactivation of MMR gene expression probably via hypermethylation may lead to inactivation of their functions which finally leads to tumor aggressiveness and the immunostaining of hMLH1 protein can be used as a prognostic factor for determining the grade of the tumor.

Keywords Colorectal cancer · hMLH1 · hMSH2 · Methylation · Immunohistochemistry

Introduction

CRC is a major cause of cancer-associated morbidity and mortality worldwide. It is the second most prevalent cancer and affects men and women almost equally [1]. The epidemiologies of CRC have been studied in Indian population [2]. However, the molecular mechanisms involved in the pathogenesis have not been investigated in Indian CRC patients.

DNA methylation is the most studied epigenetic alteration. In higher-order eukaryotes, DNA is methylated only at cytosine located 5′ to guanosine in the CpG dinucleotide. This modification has important regulatory effects on gene expression, especially when involving CpG rich areas known as CpG islands, located in the promoter region of many genes. In cancer, DNA methylation of the promoter region of a normal tumor-suppressor gene leads to the aberrant silencing of its functions.

The development of human cancer is associated with genomic instability, which causes the accumulation of genetic changes that eventually result in the conversion of normal

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cells to malignant phenotypes. The human MMR system repairs DNA replication errors or physicochemical damage. Microsatellite regions are susceptible to mutation due to slippage of DNA polymerase during DNA replication. Failure to excise these errors may lead to frameshift mutations in many target genes [3]. *MLH1*, *MSH2*, *MSH6*, and *PMS2* are major MMR genes implicated in genetic stability [4]. Genetic instability of microsatellite repeat sequences, i.e., microsatellite instability (MSI) is commonly seen in tumors associated with the hereditary nonpolyposis colorectal cancer syndrome (HNPCC) whereas it has also been observed in approximately 13 % of sporadic CRC [5-7]. In HNPCC patients, germ-line mutations have been identified in *hMLH1* and *hMSH2* genes [8]; however, somatic mutations in one of the DNA MMR genes have been reported in up to 26 % of sporadic MSI colorectal carcinoma [9-14].

Promoter region methylation has recently been demonstrated to be an important mechanism of gene inactivation in cancer [15-18]. MSI has been associated with hypermethylation of the *hMLH1* promoter region in sporadic CRC cases [19-22]. However, in a significant subset of sporadic tumors with MSI no mutations of MMR genes could be identified [23-27] and it was speculated that non-mutational mechanisms or novel genes were responsible for the defect [26, 27]. Recent studies on sporadic colorectal carcinoma have found methylation of the promoter region of *hMLH1* in 84–89 % of the tumors with MSI, whereas methylation of the promoter region of *hMSH2* seems to be rare [28-31]. However, no data regarding the inactivation of these MMR genes in colorectal cancer are available from India.

Therefore, in the present study, to understand the etiology of human colorectal cancer in Indian patients, we performed an immunohistochemical and promoter methylation analysis for the two MMR genes, *hMLH1* and *hMSH2*. We also examined the correlation between the promoter methylation and loss of expression. The relationship between methylation and immunohistochemical patterns of hMLH1/hMSH2 expression and the clinical and pathological features of colorectal adenocarcinoma as well as the correlation of these markers with clinical outcome was also analyzed in 30 CRC patients.

Material and methods

Study design

The study was a prospective analysis of 30 patients with histologically proven colorectal adenocarcinoma admitted to the Postgraduate Institute of Medical Education and Research, India between July 2004 and July 2006. Histological classification of the tumor types and stages was performed according to the World Health Organization classification method and the Tumor, Node, Metastasis System, respectively.

Information on the clinicopathological features of CRC patients was obtained from hospital records. The study was reviewed and approved by the Institute Ethics Committee which allowed us to obtain tissue samples and all pertinent follow-up information. Only patients undergoing resectional surgery were included. Patients who had history of prior chemotherapy or radiotherapy, with inoperable tumors, family history of colorectal adenocarcinoma, or those with mucinous/signet cell carcinoma were excluded from the study. Follow-up end point was September, 2007. Apart from the description of the gross features of the tumor at the time of surgery, the rest of the colon was examined for any synchronous polyp or tumor. Fresh samples from tumor, adjoining (2–5 cm from the tumor) tissue and distant mucosa (5–10 cm from the main tumor mass) were taken from the resected colorectal specimen.

For histopathological analysis, freshly removed tissue samples were immediately fixed in 10 % buffered formalin for 24 h, embedded in paraffin and histopathological assessment was carried out to determine the tumor grade and invasion. Fresh tissues were snap frozen within 10–15 min of surgical removal and stored at −80 °C till further use. Each tissue for the molecular analysis was also assessed histologically by making a crushed smear to ensure the presence of tumors and only those samples, which contained >90 % of tumor cells were included for the final analysis. Similarly, the presence of adjoining and normal colorectal mucosa was also confirmed histologically before subjecting the tissue for further analysis. The normal mucosa was used as a control in each case.

Detection of promoter methylation of *hMLH1* and *hMSH2*

For methylation analysis, all the specimens obtained during surgery procedure were treated with proteinase K and RNase. Each specimen was then subjected to DNA extraction using standard phenol–chloroform procedures [32]. The promoter methylation status of *hMLH1* and *hMSH2* gene was assessed by chemical treatment with sodium bisulfite and subsequent methylation-specific PCR (MSP) analysis according to the method of Herman et al. [33].

MSP for the *hMLH1* and *hMSH2* gene

Briefly, DNA (1 μg) in a volume of 50 μl was denatured by 0.2 M NaOH and incubated at 37 °C for 10 min; 30 μl of 10 mM hydroquinone (Sigma, St. Louis, USA) and 520 μl of 3 M Na bisulfite, pH 5.0 (Sigma, St. Louis, USA), both freshly prepared were added to denatured DNA solution. The tubes were then incubated at 50 °C/16 h. The bisulfite-treated DNA was purified using the wizard DNA clean up system (Promega, Madison, WI, USA). Modification was completed by 0.3 M NaOH treatment for 5 min at room temperature,

followed by ethanol precipitation, DNA was resuspended in distilled water and stored at −20 °C. The bisulfite modified DNA was PCR amplified by using primers specific for methylated CpG and unmethylated regions of *hMLH1* and *hMSH2* promoters. The PCR mixture contained 1× PCR buffer (16.6 mM ammonium sulfate/67 mM Tris, pH 8.8/6.7 mM MgCl$_2$/10 mM 2-mercaptoethanol), dNTPs (each at 2 mM), 10 pmol of each primer and bisulfite-treated DNA (~50 ng) or unmodified DNA (50–100 ng) in a final volume of 25 μl. Primer sequences of *hMLH1* for unmethylated reaction were 5′ TTT TGA TGT AGA TGT TTT ATT AGG GTT GT 3′ (sense) and 5′ ACC ACC TCA TCA TAA CTA CCC ACA 3′ (antisense) and for methylated reaction were 5′ ACG TAG ACG TTT TAT TAG GGT CGC 3′ (sense) and 5′ CCT CAT CGT AAC TAC CCG CG 3′ (antisense). Primer sequences of *hMSH2* for unmethylated reaction were 5′ GGT TGT TGT GGT TGG ATG TTG TTT 3′ (sense) and 5′ CAA CTA CAA CAT CTC CTT CAA CTA CAC CA 3′ (antisense) and for methylated reaction were 5′ TCG TGG TCG GAC GTC GTT C 3′ (sense) and 5′ CAA CGT CTC CTT CGA CTA CAC CG 3′ (antisense). PCR specific for unmodified DNA also included 5 % DMSO. Reactions were hot started at 95 °C for 5 min before the addition of 1.5 units of Taq DNA polymerase (Roche, GmbH, Germany). PCR amplification of the modified DNA samples consisted of 1 cycle of 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 59 °C for 1 min, and 72 °C for 1 min; and 1 cycle of 72 °C for 5 min. PCR products amplified by unmethylated and methylated primers were 124 and 115 bp respectively for *hMLH1* and 143 and 132 bp, respectively, for *hMSH2*. The PCR products were analyzed by agarose gel electrophoresis. DNA treated in vitro with CpG methylase MSssI (Sss1 methyltransferase, New England Biolabs, Ipswich, MA, USA) was used as a positive control for methylated alleles of these genes. Controls without DNA were performed for each set of PCR. Each MSP was repeated at least three times. Analysis of DNA methylation was performed in the absence of knowledge of the hMLH1 and hMSH2 expression status in all of the experiments.

Analysis of protein expression: immunohistochemistry assay

The immunohistochemistry (IHC) was performed on histology sections taken from the surgically resected specimens. All the tissues fixed in formalin were processed to make paraffin blocks. Five-micrometer sections of paraffin embedded tissues were mounted on slides coated with poly-L-lysine (Sigma, St. Louis, USA). The sections were deparaffinized by heating at 60ºC, followed by serial passages though few changes of xylenes and graded alcohol (100, 95, and 70 %). The endogenous peroxidase activity was blocked by incubating the sections with the blocking solution (0.03 % H$_2$O$_2$/methanol) for 20 min. The antigenic sites were unmasked by means of conventional household psi pressure cooker

treatment for 15 min in 10 mM citrate buffer (pH 6.0). Antibodies used were MLH1 (N-20) sc-581 (1:20) to detect hMLH1 protein and MSH2 (N-20) sc-494 (1:40; Santa Cruz Biotechnology Inc. CA, USA) to detect hMSH2 protein using a peroxidase-labeled streptavidin–biotin technique. Sections were incubated for 3 h at room temperature. Negative controls were sections treated by the same techniques but without the primary antibody. Two slides per patient sample were immunostained in separate runs. Slides were scored by a consultant pathologist using light microscopy.

The normal staining pattern for hMLH1 and hMSH2 is nuclear. Tumor cells that exhibited an absence of nuclear staining in the presence of non-neoplastic cells and infiltrating lymphocytes with nuclear staining were considered to have an abnormal pattern. Staining results were examined without knowledge of the status of the molecular analyses.

Scoring of immunostaining

The immunohistochemistry results were scored by taking percentage positivity and intensity of staining into account. A score of 0 to 3 for stain intensity was assigned: an intensity score of 0=no staining, 1=weak positivity, 2=moderate positivity and 3=strong positivity was given. The total IHC score was calculated as: IHC score=%age of positivity×intensity score.

Statistical methods

Pearson's χ^2 test was performed to analyze the relationship between *hMLH1* and *hMSH2* methylation status with their respective protein expression and with each of the clinicopathological parameters. Mann–Whitney U test was performed to determine the relationship between the expression of hMLH1 and hMSH2 and each of the clinicopathological parameters. Comparisons of the different groups were performed using the ANOVA followed by post-hoc test. The overall survival (OS) and disease-free survival (DFS) were estimated by the Kaplan–Meier method and the logrank test was used to evaluate the difference between survival of the patients with and without methylation and expression of hMLH1 and hMSH2.

Results

Clinical profile

There were 30 patients (20 males) with age range from 24–90 years (median age 56 years). Eighteen (60 %) patients had the tumor in distal colon, and 12 (40 %) in the proximal colon. None of the patients had a synchronous adenoma or carcinoma. The median length of the tumors was 5 cm (range 2–10 cm). There were four patients (13.3 %) in stage I, 18 (60 %)

in stage II, 6 (20 %) in stage III and 2 (6.6 %) in stage IV disease. Metastasis was found in eight patients with distant metastasis in liver ($n=2$) and lymph node ($n=6$). None of the patients had a family history of CRC or any other kind of malignancy. All tumors were adenocarcinomas and on histology, 6 were well differentiated, 21 moderately differentiated, and 3 poorly differentiated adenocarcinomas.

Promoter methylation analysis of *hMLH1* and *hMSH2*

Methylation status of *hMLH1* and *hMSH2* genes was evaluated in 30 colorectal tumors, adjoining and normal mucosa. Out of 30 cases of CRC, promoter methylation of *hMLH1* and *hMSH2* genes was observed in 15 (50 %) and 3 (10 %) tumors, respectively. As the tumor samples also contain normal cells, so the amplification of the unmethylated sequence was observed in all tumors. In colorectal adjoining and normal mucosa only unmethylated band of *hMLH1* and *hMSH2* genes were present. No promoter methylation of *hMLH1* and *hMSH2* genes was observed in adjoining and normal mucosa (Fig. 1). Combination of methylation of *hMLH1* with *hMSH2* genes was observed in 2 (6.6 %) tumors.

Protein expression of hMLH1

In colorectal adenocarcinoma 26 (86.6 %) tumors showed hMLH1 protein expression. Complete loss of hMLH1 protein expression was observed in 4 (13.3 %) tumors. Out of 26 positively stained tumors hMLH1 expression was observed weak in 15, moderate in 10 and strong in 1 tumor (Fig. 2a–b). hMLH1 protein showed immunoreactivity in all the 30 adjoining and normal mucosa (Fig. 2c–d). The expression of hMLH1 protein was weak in 6, moderate in 20 and strong in 4 cases of adjoining mucosa. There was a significant difference

Fig. 1 Depicts promoter methylation analysis of *hMLH1* and *hMSH2* in representative cases of colorectal cancer (CRC) in normal (*N*), adjoining (*A*) and tumor (*T*). hMLH1 (*U*) shows unmethylated PCR products for hMLH1 gene and hMLH1 (*M*) shows methylated PCR products for hMLH1gene. Similarly, hMSH2 (*U*) shows Unmethylated PCR products for hMSH2 gene and hMSH2 (*M*) shows methylated PCR products for hMSH2 gene

in the expression of hMLH1 in adjoining and normal mucosa as compared to tumor at nuclear level ($p=0.001$).

Protein expression of hMSH2

hMSH2 protein showed immunoreactivity in 28 tumors, while loss of expression was observed in 2 tumors (6.6 %). Out of 28 positively stained tumors, weak expression was observed in 10 tumors, moderate in 17, and strong in 1 tumor (Fig. 3a–b). In adjoining and normal mucosa, positive immunostaining was present in all the 30 samples (Fig. 3c–d). Weak expression of hMSH2 protein was observed in 8, moderate in 17, and strong in 5 samples. There was a significant difference in the expression of hMSH2 protein between tumor and normal mucosa at nuclear level ($p=0.029$).

Clinicopathological correlation with promoter methylation and expression

Methylation and expression of the *hMLH1* and *hMSH2* genes had no correlation with clinicopathological factors ($p>0.05$) including, TNM stage, Duke's stage, smoking/alcohol consumption, metastasis, tumor site, pre- and postoperative serum CEA levels, age, etc. A significant correlation was observed between the histological grade of the tumor and expression of *hMLH1* gene ($p<0.05$) (Fig. 4).

Correlation of promoter methylation with the protein expression

The protein expression of hMLH1 was compared to promoter methylation of *hMLH1* and the results are summarized in Table 1. Out of the 26 colorectal adenocarcinoma positive for hMLH1 protein expression, 11 had methylation in the promoter region of *hMLH1* gene. Out of 11 methylated samples, 7 had reduced expression and 4 had moderate expression. However, four tumors totally negative for hMLH1 expression also had methylation in the promoter region. There was a significant effect of *hMLH1* gene methylation on the expression of hMLH1 protein ($p=0.002$).

Similarly, the protein expression of hMSH2 was compared with respect to their respective gene methylation and the results are summarized in Table 2. In case of 28 colorectal adenocarcinomas positive for hMSH2 protein expression, 1 had methylation in the promoter region of *hMSH2* gene. However, two tumors totally negative for hMSH2 expression also had methylation in their promoter regions. However, one case showed a loss of both hMLH1 and hMSH2 expression.

Survival analysis

A follow-up data of the patients enrolled for this study was obtained for 29 patients. At the last follow-up (September,
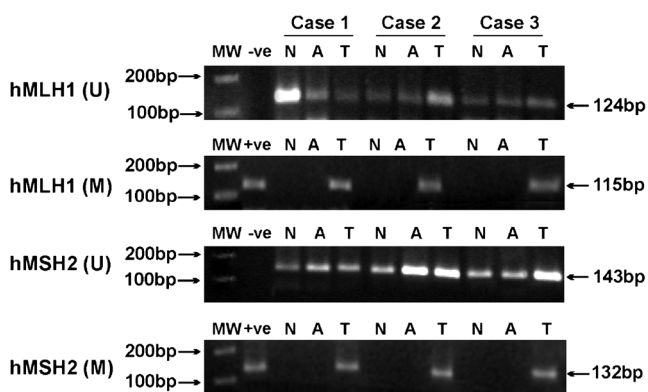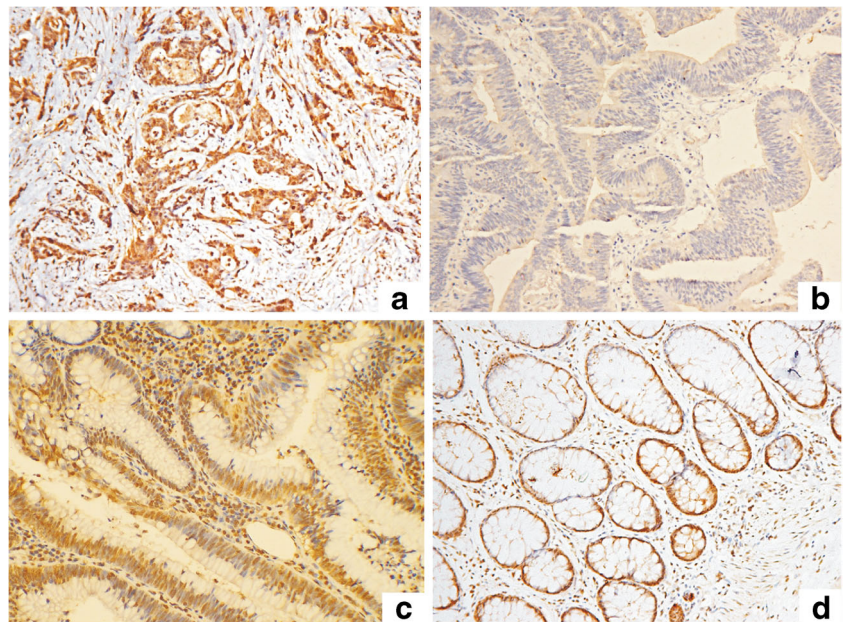
Fig. 2 Depicts protein expression of hMLH1in representative case of colorectal cancer (CRC). **a** and **b** show protein expression of hMLH1 in tumor tissues; **c** and **d** show protein expression of hMLH1 in adjoining and normal mucosa

2007), four patients (13.3 %) died, 4 patients (13.3 %) showed the recurrence of disease with distant metastasis in 2 patients (6.6 %) and 21 patients (70 %) were alive without disease.

The association of methylation and expression of hMLH1 and hMSH2 with disease-free and overall survival was analyzed. Disease-free survival was defined as the time from the date of surgical resection of the tumor to the date of recurrence of the disease. The median follow-up period was 27 months with a range of 8–39 months (mean=25.5±8.14). Overall survival was defined as the time from the date of diagnosis of CRC to the date of last follow-up. The median follow-up

period was 27.5 months with a range of 8–39 months (mean= 26.36±8.19). By Kaplan–Meier logrank survival analysis it was found that the methylation and expression of *hMLH1* and *hMSH2* has no effect on the survival of the patients with colorectal adenocarcinomas ($p > 0.05$).

Discussion

There are two major mechanisms of gene inactivation. First is the genetic mechanism, e.g., the aberration of DNA structure

Fig. 3 Protein expression of hMLH1in representative case of colorectal cancer (CRC). **a** and **b** show protein expression of hMSH2 in tumor tissues; **c** and **d** show protein expression of hMSH2 in adjoining and normal mucosa
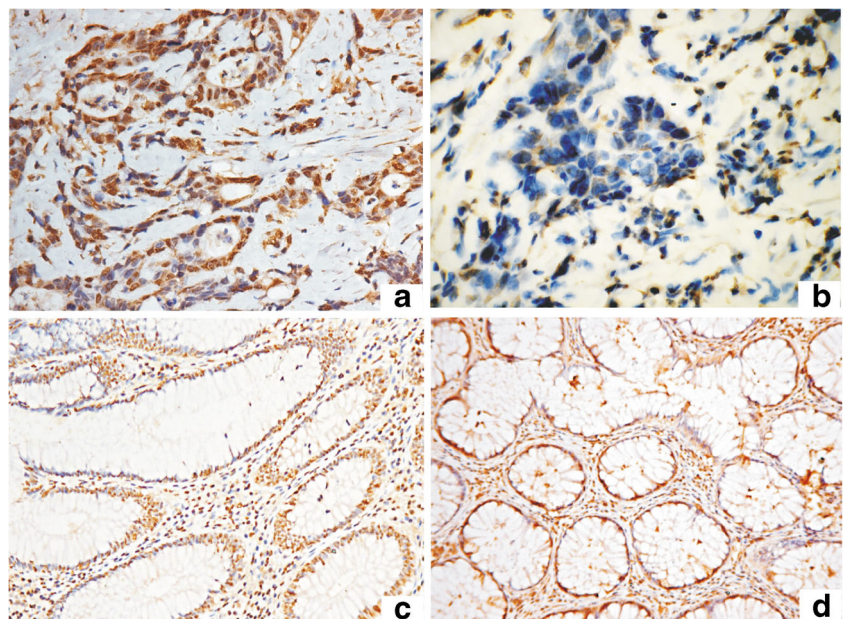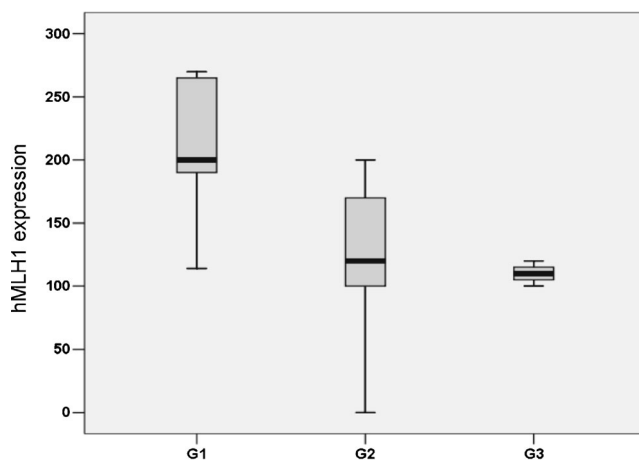
Fig. 4 Depicts correlation of hMLH1 protein expression and histological grade of tumor in representative case of colorectal cancer (CRC)

such as homozygous deletion or intragenic mutation resulting in the gene inactivation and second is the epigenetic mechanism, e.g., the methylation at position 5′ of cytosine residue leading to the lack of gene expression, while the structure and the product of the gene remain unchanged.

The human DNA repair system plays an important role in reducing mutations and maintaining genomic stability. The MMR genes *hMLH1* and *hMSH2* are integral components of the DNA mismatch repair pathway. Defective DNA mismatch repair is most commonly associated with the functional loss of *hMLH1* and *hMSH2* genes and results in the mutator phenotype characterized by MSI in CRC. Aberrant methylation, which can result in the transcriptional silencing of the target gene, was frequently found in the *hMLH1* and *hMSH2* alterations in colorectal cancer. In the present study, MSP assays were used to examine the promoter methylation of *hMLH1* and *hMSH2* genes in tumor, adjoining and normal mucosa. The methylation frequencies of *hMLH1* were observed in 50 % and of *hMSH2* in 10 % of tumors, whereas no promoter methylation of *hMLH1* and *hMSH2* genes was observed in adjoining and normal mucosa. The hypermethylation of *hMSH2* gene was quite low in this population study, consistent with the previous study in Asian population [29]. However, majority of the studies have reported absence of promoter methylation of *hMSH2* gene [28, 30]. In a study, Herman et al. [30] reported hypermethylation of *hMLH1* in 84 % of MSI+ sporadic CRCs and suggested that methylation of the *hMLH1* promoter is an epigenetic event that plays a causal role in the MMR defect in many MSI+ cancers. In another study, Miyakura et al. reported methylation of *hMLH1* gene promoter in 88.9 % of CRCs in Japanese [31]. The difference in frequency of methylation may be due in part to differences in the type or stage of tumors analyzed in the contrasting studies. It is also possible that geographical/environmental factors also account for the frequent alterations of *hMLH1* and *hMSH2*. Majority of the studies reported

methylation of *hMLH1* and *hMSH2* gene and correlated with MSI status but in the present study MSI was not analyzed.

Further, the correlation of *hMLH1* and *hMSH2* gene methylation with different clinicopathological factors was analyzed and it was observed that hypermethylation of *hMLH1* and *hMSH2* gene did not correlate with age, metastases, pre- and postoperative serum CEA level, stage, and tumor size. A significant positive correlation between the methylation of *hMLH1* and the histological grade of the tumor ($p = 0.040$) was observed. Frequent methylation of *hMLH1* gene was found to be present in poorly differentiated colorectal carcinoma suggesting that inactivation of the gene due to methylation leads to tumor aggressiveness.

The immunohistochemical analysis of hMLH1 and hMSH2 protein was also done to evaluate the prognostic significance of MMR status in tumor tissues, adjoining and normal mucosa of sporadic CRC patients. Almost all the tumors expressed both hMLH1 and hMSH2 proteins. Complete loss of hMLH1 expression was observed in four tumors (13.3 %) and of hMSH2 in two tumors (6.6 %) whereas 50 % tumors showed reduced expression of hMLH1 and 33.3 % showed reduced expression of hMSH2 protein. These results are consistent with other studies [34]. The normal nuclear staining pattern of hMLH1 and hMSH2 was observed in majority of adjoining and normal mucosa whereas some cases of adjoining mucosa showed reduced expression of hMLH1 and hMSH2 protein. Hammed et al. [35] have reported that 60 % of the colorectal tumors demonstrated normal expression of both hMLH1 and hMSH2 protein and 27 % tumors did not express hMLH1 and 13 % hMSH2 proteins. In another study, Lanza et al. [34] reported that MSS or MSI-L tumors showed normal hMLH1/hMSH2 expression by immunohistochemistry whereas MSI-H carcinomas showed complete loss of hMLH1/hMSH2 protein expression. However, in the present study, a highly significant correlation was found between *hMLH1* and *hMSH2* gene methylation and loss/reduced protein expression ($p < 0.01$) in tumors from colorectal cancer patients, suggesting that promoter methylation is the predominant mechanism by which these two genes are silenced and might affect the protein expression in sporadic CRC. In addition, some tumors also expressed hMLH1 even with the methylation in their promoter regions. This may be due to the reason that in such cases only one allele is methylated and the remaining unmethylated allele would be expected to produce a sufficient amount of hMLH1 protein, resulting in proficient mismatch repair. Alternatively, it might be possible that methylation of other CpG sites is important in silencing the *hMLH1* gene or otherwise full methylation in a wide region of *hMLH1* promoter is necessary for gene silencing of *hMLH1*. However, few cases also showed reduced expression of hMSH2 protein without the promoter methylation in their promoter region which indicates that methylation of the specific sites examined may be important but may not

Table 1 Comparison of hMLH1 gene methylation with hMLH1 protein expression (score 0=no intensity, 100–200=weak intensity, 200–300=moderate intensity, more than 300=strong intensity)

Sample no.	Tumor		Adjoining		Normal	
	hMLH1 Protein expression	*hMLH1* Methylation	hMLH1 Protein expression	*hMLH1* Methylation	hMLH1 Protein expression	*hMLH1* Methylation
S-1	160	−	180	−	230	−
S-2	0	+	120	−	180	−
S-3	114	+	190	−	240	−
S-4	100	+	190	−	200	−
S-5	120	+	240	−	250	−
S-6	182	−	200	−	200	−
S-7	110	+	190	−	200	−
S-8	200	−	200	−	240	−
S-9	110	+	250	−	250	−
S-10	120	+	150	−	200	−
S-11	0	+	100	−	180	−
S-12	125	+	180	−	180	−
S-13	100	+	100	−	150	−
S-14	200	−	200	−	250	−
S-15	150	−	150	−	200	−
S-16	200	−	200	−	250	−
S-17	140	−	200	−	250	−
S-18	200	−	210	−	250	−
S-19	90	−	100	−	210	−
S-20	100	+	200	−	220	−
S-21	200	−	240	−	250	−
S-22	100	+	200	−	240	−
S-23	100	+	150	−	200	−
S-24	200	−	300	−	300	−
S-25	0	+	110	−	300	−
S-26	190	−	200	−	210	−
S-27	180	−	240	−	260	−
S-28	160	−	200	−	200	−
S-29	190	−	300	−	300	−
S-30	0	+	200	−	200	−

be sufficient for gene inactivation and a broad analysis of all the CpG sites are required.

Further, the correlation of hMLH1 and hMSH2 protein expression with different clinicopathological factors was analyzed and it was observed that loss/reduced expression of hMLH1 and hMSH2 protein did not correlate with any of the clinicopathological factors examined except the degree of differentiation of the tumor. There was significant association between hMLH1 protein expression and tumor grade. The expression of hMLH1 protein was reduced in poorly differentiated adenocarcinomas as compared to the well-differentiated adenocarcinomas. A similar observation was reported with the methylation of *hMLH1* gene and tumor

grade which suggests that loss/reduction in the expression of hMLH1 as a result of hypermethylation increases as the grade of the tumor increases. So, the frequent occurrence of promoter methylation of the *hMLH1* gene and loss/reduced expression of hMLH1 protein in poorly differentiated carcinomas indicates its potential use as a diagnostic marker in CRC. This should be confirmed in larger patient subsets.

Finally, the clinical implications of various molecular changes in the patient's cohort was studied to establish, whether the observed changes affect the disease free survival and the overall survival. In the present study, no correlation could be established for the methylation and expression of *hMLH1* and *hMSH2* genes to the survival using univariate analysis.

Table 2 Comparison of hMSH2 gene methylation with hMSH2 protein expression (score 0=no intensity, 100–200=weak intensity, 200–300=moderate intensity, more than 300=strong intensity)

Sample no.	Tumor		Adjoining		Normal	
	hMSH2 Protein expression	*hMSH2* Methylation	hMSH2 Protein expression	*hMSH2* Methylation	hMSH2 Protein expression	*hMSH2* Methylation
S-1	110	−	160	−	180	−
S-2	100	−	190	−	200	−
S-3	80	+	120	−	160	−
S-4	0	+	140	−	190	−
S-5	200	−	250	−	250	−
S-6	190	−	220	−	240	−
S-7	80	−	120	−	190	−
S-8	200	−	230	−	260	−
S-9	200	−	300	−	300	−
S-10	100	−	180	−	190	−
S-11	0	+	170	−	190	−
S-12	100	−	200	−	270	−
S-13	140	−	250	−	300	−
S-14	100	−	200	−	250	−
S-15	90	−	280	−	280	−
S-16	200	−	270	−	300	−
S-17	50	+	100	−	170	−
S-18	90	+	100	−	160	−
S-19	200	−	200	−	250	−
S-20	140	−	210	−	220	−
S-21	90	−	120	−	230	−
S-22	200	−	250	−	250	−
S-23	100	−	300	−	300	−
S-24	190	−	220	−	300	−
S-25	180	−	200	−	220	−
S-26	170	−	200	−	200	−
S-27	200	−	300	−	300	−
S-28	200	−	290	−	300	−
S-29	200	−	300	−	300	−
S-30	300	−	300	−	300	−

All reported studies have conserved the adenoma→carcinoma sequence and reported the presence of genetic alterations in adenomas, a precursor lesion that finally develops to carcinoma. But only a very small number of the Asian patient population have adenomas; therefore, the present study included adjoining mucosa so as to determine the initial changes in CRC. But in case of adjoining mucosa, no major changes were observed which suggested that there are no changes near the tumor region and the process of tumorigenesis is restricted to a limited area.

An important aspect of this study is that for the first time, two major MMR genes have been analyzed at both genetic and expression level in CRC in relation to pathophysiology and prognosis in small Indian patient cohort. The limitation of the study is the small size of the cohort. As this was the pilot study on Indian population, we need more patients from different parts of the country to validate our findings.

In conclusion, our study demonstrated that majority of CRC tissues expressed MMR proteins hMLH1 and hMSH2 and the low or reduced expression of these proteins among CRC tissues probably via hypermethylation may leads to inactivation of their functions which finally leads to tumor aggressiveness and the immunostaining of hMLH1 protein can be used as a prognostic factor for determining the grade of the tumor. Although the cases in the present study were not enough to draw definite conclusions, however, it may be

suggested that the promoter hypermethylation of *hMLH1 and hMSH2* genes may be one of the causes of inactivation of these genes in sporadic CRC.

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