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Microsatellite Instability in Inflammatory Bowel Disease-associated Neoplastic Lesions Is Associated with Hypermethylation and Diminished Expression of the DNA Mismatch Repair Gene, *hMLH1*[1]

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ABSTRACT

Twelve to 15% of sporadic colorectal cancers display defective DNA mismatch repair (MMR), manifested as microsatellite instability (MSI). In this group of cancers, promoter hypermethylation of the MMR gene *hMLH1* is strongly associated with, and believed to be the cause of, MSI. A subset of colorectal neoplastic lesions arising in inflammatory bowel disease (IBD) is also characterized by MSI. We wished to determine whether *hMLH1* hypermethylation was associated with diminished hMLH1 protein expression and MSI in IBD neoplasms. We studied 148 patients with IBD neoplasms, defined as carcinoma or dysplasia occurring in patients with ulcerative colitis or Crohn's disease. MSI was evaluated using multiplex fluorescent PCR to amplify loci *D2S123*, *BAT-25*, *BAT-26*, *D5S346*, and *D17S250* in all cases. Lesions were characterized as high-frequency MSI (MSI-H) if they manifested instability at two or more loci, low-frequency MSI (MSI-L) if unstable at only one locus, or MS-stable (MSS) if showing no instability at any loci. Methylation-specific PCR was performed to determine the methylation status of the *hMLH1* promoter region. hMLH1 protein expression was also evaluated by immunohistochemistry. Thirteen (9%) of 148 neoplasms arising in IBD were MSI-H, comprising 11 carcinomas and 2 dysplastic lesions. Sixteen additional lesions (11%) were MSI-L, comprising 11 carcinomas and 5 dysplastic lesions. The remaining 118 neoplasms (80%) were MSS. Six (46%) of 13 MSI-H, 1 (6%) of 16 MSI-L, and 4 (15%) of 27 MSS lesions showed *hMLH1* hypermethylation ($P = 0.013$). Diminished hMLH1 protein expression in neoplastic cell nuclei relative to surrounding normal cell nuclei was demonstrated immunohistochemically in four of four (100%) hypermethylated lesions tested. In IBD neoplasia, *hMLH1* promoter hypermethylation occurs frequently in the setting of MSI, particularly MSI-H. Furthermore, *hMLH1* hypermethylation and MSI are strongly associated with diminished hMLH1 protein expression in IBD neoplasms. These findings suggest that *hMLH1* hypermethylation causes defective DNA MMR in at least a subset of IBD neoplasms.

INTRODUCTION

CRC[4] is the most dreaded complication of IBD. The development of CRC is the most serious long-term complication faced by patients with longstanding extensive ulcerative colitis and Crohn's colitis, with an incidence 20-fold higher and an average age of onset 20 years younger than CRC in the general population (1). It is generally accepted that this increased risk does not begin until 8–10 years after the diagnosis of IBD. Thereafter, it increases by ~0.5–1.0% per year (2–4). The most significant predictor of the risk of malignancy in IBD is the presence of dysplasia on colonic biopsies. Colonoscopy with biopsies for dysplasia can help stratify patients into risk groups. However, the effectiveness of surveillance programs has been questioned (2). The discovery of new molecular alterations may lead to the development of more accurate screening biomarkers (5).

MSI comprises length mutations in tandem oligonucleotide repeats, which occur in a large subset of human tumors (6–9). This type of mutation is believed to be caused by deficient DNA MMR (10–13). Twelve to 15 of sporadic CRCs display defective DNA MMR, manifested as MSI (14). The underlying cause of MSI in CRC has been the subject of intensive research. Mutations in known MMR genes have been described in kindreds with CRCs meeting the Amsterdam criteria for the diagnosis of hereditary nonpolyposis colon cancer (15). Initially, germ line mutations in the *hMSH2* gene were described; subsequently, mutations were also reported in other MMR genes, namely *hMLH1*, *hPMS1*, *hPMS2*, *hMSH3* (*DUG*), and *hMSH6* (*GTBP*) (16–22). However, the majority of sporadic CRCs with MSI do not harbor mutations in known MMR genes (14, 23). In sporadic CRCs, hypermethylation involving the promoter region of the MMR gene *hMLH1* is strongly associated with, and widely believed to cause, MSI. Moreover, in MSI-positive sporadic gastric and endometrial tumors, *hMLH1* gene promoter hypermethylation is very frequent and is often accompanied by down-regulated or absent *hMLH1* gene expression (24–27). This type of epigenetic gene inactivation is not limited to *hMLH1*. Hypermethylation of normally unmethylated CpG islands in the promoter regions of multiple tumor suppressor genes, including *p16*, *p15*, *VHL*, *E-cadherin*, *GSTP1*, and *MGMT*, suggests an alternative mechanism of gene inactivation (28, 29). A subset of colorectal neoplasms arising in IBD is also characterized by MSI (30, 31). Therefore, we sought to ascertain whether *hMLH1* hypermethylation occurred, and whether it was associated with diminished hMLH1 protein expression and MSI, in IBD neoplasms.

MATERIALS AND METHODS

We defined IBD neoplasia as any colorectal dysplasia or carcinoma arising in patients with ulcerative colitis or Crohn's colitis. One hundred forty-eight samples from patients with IBD neoplasia were analyzed. Samples were obtained consecutively from patients presenting to the Mt. Sinai University Medical Center after informed consent, in compliance with internal institutional board review. Tissues were stored at −180°C until study. DNA was extracted using a previously described technique (32). Parallel morphological sections were used for diagnosis, and all of the dysplastic as well as the

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[4] The abbreviations used are: CRC, colorectal cancer; hMLH1, human mut-L homologue 1; IBD, inflammatory bowel disease; MMR, mismatch repair; MSI, microsatellite instability; MSP, methylation-specific PCR; MSI-H, high-frequency MSI; MSI-L, low-frequency MSI; MSS, stable microsatellite.

cancerous lesions were grossly visible to the pathologist (N. H.). Microdissection was not performed.

MSI. The MSI status of all of the 148 neoplasms was determined by PCR using fluoromer-labeled primers to amplify markers *BAT25*, *BAT 26*, *D17S250*, *D5S346*, and *D2S123* (33). PCR was performed in three separate tubes for markers *D17S250*, *BAT25*, and *BAT26*. Markers *D5S346* and *D2S123* were multiplexed in the same reaction tube. Primers were fluorescently labeled as follows: BAT26 was labeled with a green dye, tet; BAT25 with a yellow dye, hex; and D5S346, D2S123 and D17S250 were all labeled with a blue dye, fam. PCRs were carried out in 10-μl volumes containing 60 ng of genomic DNA, 1 pmol of each primer, 50 mM KCl, 10 mM Tris, 0.4 mM each dNTP, 1.5 mM $MgCl_2$, and 0.5 units of Taq DNA polymerase (Promega, Madison, WI). PCRs consisted of an initial denaturation step at 94°C for 4 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension step at 72°C for 6 min. The annealing temperature varied among primer sets but was between 50 and 60°C. Simultaneous gel electrophoresis of PCR products was performed on 5.25% Long Ranger gels (Bio-Rad, Hercules, CA) containing 6 M urea. Samples were loaded onto 96-lane gels and electrophoresed on an ABI Prism 377 automated DNA sequencer (Perkin-Elmer, Norwalk, CT). Gels were scored in two steps using separate programs. The first program, Genescan (ABI), checked for consistency across all lanes. Consistency was also checked manually. The second program, Genotyper (ABI), assigned allele sizes and derived peak heights in arbitrary fluorescent units. Peak heights were then evaluated by hand, with the color and size of each peak identifying the product being evaluated and the presence of MSI.

Lesions were characterized as MSI-H if they manifested instability at two or more loci, MSI-L if unstable at only one locus, or MSS if showing no instability at any loci.

MSP. *hMLH1* hypermethylation was determined in 13 MSI-H, 16 MSI-L, and 27 MSS lesions. DNA methylation patterns in the *hMLH1* promoter were determined by MSP as described previously (34). MSP distinguishes unmethylated from hypermethylated alleles based on sequence alterations produced by bisulfite treatment of DNA, which converts unmethylated, but not methylated, cytosines to uracil, and subsequent PCR using primers specific to either methylated or unmethylated DNA. Briefly, 1 μg of genomic DNA was denatured by treatment with NaOH and was modified by sodium bisulfite. DNA samples were purified using Wizard DNA purification resin (Promega), again treated with NaOH, precipitated with ethanol, and resuspended in water. PCR was then performed using the primer pairs described below under the following conditions: the PCR mix contained 10× PCR buffer [16.6 mM ammonium sulfate, 67 mM Tris (pH 8.8), 6.7 mM $MgCl_2$, and 10 mM 2-mercaptoethanol], dNTPs (each at 1.25 mM), primers (300 ng each per reaction), and bisulfite-modified DNA (50 ng) in a final volume of 50 μl. Reactions were hot-started at 95°C for 5 min before the addition of 1.25 units of *Taq* polymerase (Life Technologies, Inc.). Amplification was carried out in a Hybaid OmniGene temperature cycler (Hybaid, Middlesex, United Kingdom) for 35 cycles (30 s at 95°C, 30 s at 59°C, then 30 s at 72°C), followed by a final 4-min extension at 72°C. Control PCRs lacking genomic DNA were performed for each set of reactions. DNA from colon cancer cell line SW48, which is completely hypermethylated at the *hMLH1* locus, was used as a positive control (23). DNA from normal lymphocytes served as a negative control for hypermethylated *hMLH1*. Ten μl of each PCR reaction product were directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Primer sequences of *hMLH1* for the 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 the methylated reaction, they were 5′-ACG TAG ACG TTT TAT TAG GGT CGC-3′ (sense) and 5′-CCT CAT CGT AAC TAC CCG CG-3′ (antisense).

Immunohistochemistry. Frozen tissue was thawed on ice, fixed in 10% formalin, and embedded in paraffin blocks. Five-μm sections were mounted on glass slides. Sections were then deparaffinized with xylene for 30 min and rehydrated using graded ethanols. Antigen retrieval was performed using a heat-induced epitope retrieval method (35). Immunoperoxidase staining using diaminobenzidine as chromogen was performed with the TechMate 1000 automatic staining system (Ventana, BioTek Solutions, Tucson, AZ). Mouse monoclonal antibody to the *hMLH1* gene product was used at 1:300 dilution (PharMingen, San Diego, CA). Staining of tumor cells was evaluated as present or absent in stained slides.

Statistical Correlations. Analyses were performed using Statview 4.5 and superANOVA software for the Macintosh (SAS Institute Inc., Cary, NC). Two-by-two table contingency analyses were performed using a two-tailed Fisher's exact test because some numerical values were less than 5.

RESULTS

MSI. Of the 148 neoplasms compared with matched normal control tissue, 13 were MSI-H (8.78%), 16 (11%) were MSI-L, and 118 (80%) were MSS. Results of these MSI studies are summarized in Table 1. Interestingly, 13 (81%) of 16 MSI-L lesions showed only mononucleotide instability, whereas 11 (85%) of 13 MSI-H lesions showed dinucleotide instability (3 with dinucleotide instability alone, and 8 with both dinucleotide and mononucleotide instability; $P = 0.001$ for MSI-L *versus* MSI-H, Fisher's exact test, two-tailed).

MSP. Results of *hMLH1* promoter hypermethylation assays *versus* MSI studies in IBD neoplasms are summarized in Table 1 and illustrated in Fig. 1. *hMLH1* promoter methylation was determined in all of the MSI-H or MSI-L specimens and in 27 of the MSS lesions.

Hypermethylation of the *hMLH1* promoter occurred in 6 (46%) of 13 MSI-H cases, whereas only 1 (6%) of 16 MSI-L cases and 4 (15%) of 27 MSS cases showed this finding. When MSI-H cases were compared with MSI-L and MSS cases together, MSI-H was significantly associated with *hMLH1* hypermethylation ($P = 0.013$, Fisher's exact test, two-tailed). The difference in hypermethylation rates between MSI-L and MSS cases was not significant ($P = 0.635$, Fisher's exact test, two-tailed). Among the MSI-H lesions, there were two high-grade, one mixed low- and high-grade, and one low-grade dysplasia and two moderately differentiated carcinomas in the hypermethylated subgroup, whereas there were five moderately differentiated carcinomas and one low- and one high-grade dysplasia in the unmethylated subgroup. This difference in frequency of dysplastic lesions was not statistically significant ($P = 0.13$, Fisher's exact test, two-tailed. Moreover, 5 of 15 MSI-L unmethylated lesions were dysplasias *versus* 0 of 1 in the MSI-L hypermethylated subgroup. Similarly, 6 of 23 MSS unmethylated lesions were dysplasias *versus*

Table 1 *MSI and hMLH1 hypermethylation in IBD-neoplasia*

Sample numbers[a]	Total	MSI status[b]	*hMLH1* methylation status[c]	hMLH1 protein
H4D1, H4D3, H81T, H75D, H85T, H109T	6	MSI-H	Hypermethylated	Absent in 4/4 lesions
H8T, H11T, H41T, H57T, H69T, H69D, H83T	7	MSI-H	Unmethylated	
H70T	1	MSI-L	Hypermethylated	
H9D5, H9D6, H9D7, H16T, H29T, H30D, H30T3, H32T, H35T, H52T, H53T2, H80T, H82T, H84T, H107T	15	MSI-L	Unmethylated	Present in 2/2 lesions
H30T1, H30T2, H31T2, H36T1	4	MSS	Hypermethylated	
H4T, H22T1, H22T2, H23T, H24T, H26D, H27T1, H27T2, H28T, H28D, H26T1, H26T2, H31T1, H31D, H32D, H36T2, H36T3, H36T4, H37T, H38T, H39T, H40T, H42T2	23	MSS	Unmethylated	

[a] "T" and "D" in sample numbers denote tumors or dysplasias, respectively.

[b] Tumors were characterized as MSI-H if they manifested instability at two or more loci (or greater than 30% of loci if more than five loci were tested), MSI-L if unstable at only one locus (or less than 30% of loci if more than five loci were tested), and MSS if showing no instability at any loci.

[c] $P = 0.013$ for methylation status of MSI-H *versus* [MSI-L + MSS] (Fisher's exact test, two-tailed).

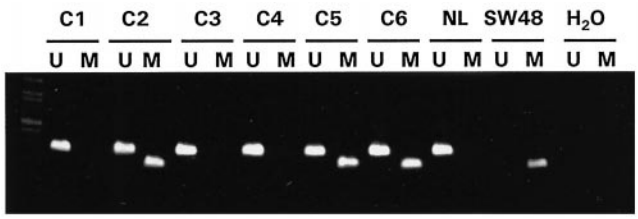

Fig. 1. Hypermethylation of the *hMLH1* promoter region CpG island in human IBD neoplasms. The presence of PCR product in *lanes marked U* indicates unmethylated *hMLH1*; product in *lanes marked M* indicates hypermethylated *hMLH1*. All of the primary tumors display unmethylated bands presumably contributed by contaminating normal cells. Molecular size marker lane is *at left*. C1-C6 represent 6 of the 13 MSI-H IBD neoplasms. Samples C2, C5, and C6 are hypermethylated, whereas samples C1, C3, and C4 are unmethylated. Known hypermethylated SW48 cancer cells serve as positive controls, whereas unmethylated normal lymphocytes (*NL*) serve as negative controls. H_20, water control (lacking genomic DNA).

0 of 4 the MSS hypermethylated subgroup. Thus, early lesions were no more frequent in the hypermethylated than in the unmethylated groups, and tumor grades did not correlate with methylation status.

Immunohistochemistry. Six cases, comprising four MSI-H *hMLH1* hypermethylated (H75T, H81T, H85T, and H109T) and two MSI-L *hMLH1* unmethylated (H80T and H84T) neoplasms, were tested for hMLH1 nuclear protein expression using immunohistochemistry. All four of the *hMLH1* hypermethylated tumors showed diminished hMLH1 expression in tumor cell nuclei relative to normal cell nuclei in the same sections. The two unmethylated samples showed abundant expression of hMLH1 nuclear protein. Representative immunohistochemical data are displayed in Fig. 2.

DISCUSSION

The present study suggests that a significant subgroup of IBD neoplasms manifesting MSI-H demonstrate hypermethylation of the *hMLH1* gene promoter, and that this hypermethylation is associated with a lack of hMLH1 protein expression. These data are consistent with previous observations showing frequent *hMLH1* promoter hypermethylation and gene silencing in MSI-H sporadic non-IBD CRCs, gastric cancers, and endometrial cancers (24–27, 36, 37).

Although 6 of 13 MSI-H IBD neoplasms were hypermethylated, a roughly equivalent proportion (7of 13) of MSI-H IBD neoplasms were not hypermethylated in the *hMLH1* promoter. Notwithstanding the relatively small number of MSI-H cases, this prevalence of unmethylated cases still looms large when one considers that a minority of sporadic CRCs contains known MMR gene mutations (14). MSI in these seven cases could have derived from mutations in any of the known MMR genes: we did not assay for these mutations (14–22, 38). Furthermore, even in sporadic CRCs with MSI, *hMLH1* hypermethylation rates are approximately 80%. Thus, MMR gene mutations or other mechanisms must underlie MSI in the remaining 20% of these tumors.

If MSI-L tumors are considered as lying along a continuum leading to full-blown MSI, the rarity of *hMLH1* promoter hypermethylation in MSI-L IBD neoplasms can be interpreted to mean that MSI-L CRCs biologically resemble MSS cases; *i.e.*, a single microsatellite mutation may reflect a random event unrelated to any underlying MMR defect, and, thus, may be unrelated to *hMLH1* hypermethylation. On the other hand, *hMLH1* hypermethylation in 1 of 16 MSI-L and 4 of 27 MSS lesions may reflect "early" *hMLH1* gene inactivation, before development of the full-blown MSI-H phenotype: these cases may have developed MMR deficiency too recently to have accumulated large numbers of microsatellite alterations. Data supporting this latter explanation include *hMLH1* hypermethylation in MSI-L primary gastric carcinomas (26). A third explanation for the rarity of hypermethylation seen in our MSI-L and MSS lesions is that only one allele was methylated in these tumors. *i.e.*, hypermethylation of one allele could have resulted in hypermethylation by MSP but not in MMR deficiency. The CRC cell line HT-29, which shows only partial methylation of hMLH1 and is MMR-proficient (36), supports this last possibility. And finally, it is also possible that the mechanisms underlying MSI-L differed from those underlying MSI-H: exclusively mononucleotide instability predominated in MSI-L lesions, whereas mixed dinucleotide and mononucleotide instability prevailed in MSI-H lesions, and this difference was statistically significant. That is, the MMR genes correcting dinucleotide instability may differ from those repairing mononucleotide replication errors.

It has long been believed that chronic inflammatory states such as that caused by IBD predispose to cancer development. Mechanisms accounting for this predisposition to malignancy have been proposed

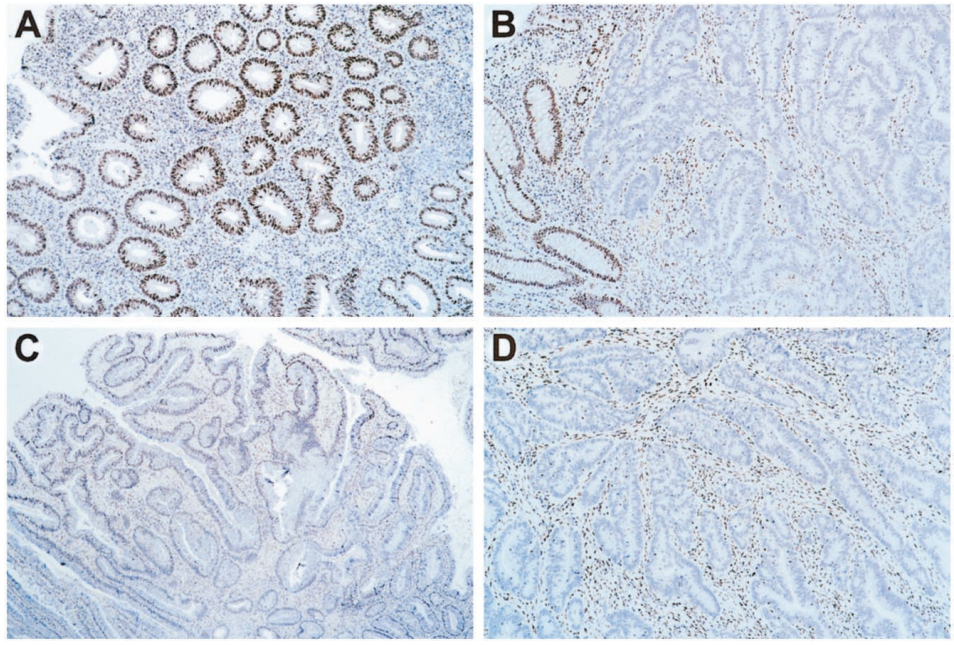

Fig. 2. hMLH1 protein expression assessed by immunohistochemical staining in hypermethylated MSI-high IBD neoplasms. *A*, low-power view of normal colonic mucosa, expressing abundant brown-staining hMLH1 protein in epithelial cell nuclei. *B*, low-power view of MSI-H IBD neoplasm with absent hMLH1 protein in neoplastic cell nuclei but abundant protein in adjacent normal glandular and stromal cell nuclei. *C* and *D*, low-power views of two different MSI-H IBD neoplasms with hypermethylation of *hMLH1*, showing absent hMLH1 protein in neoplastic cell nuclei.

(39, 40). In this context, the possible link between DNA MMR and chronic inflammation has received considerable attention (41–43). There is a strong association between chronic inflammation caused by the bacterium *Helicobacter pylori* and gastric carcinogenesis (44, 45). In fact, among sporadic cancers, the highest reported MSI prevalence occurs in those of the stomach (8, 45–48). Furthermore, MSI is associated with the intestinal type of gastric cancer (48), which is most strongly associated with *H. pylori* infection (48–51). Thus, chronic infection and inflammation have been associated with both carcinogenesis and MSI.

MSI has also been documented in the nonneoplastic inflammatory lesions, pancreatitis and ulcerative colitis (42, 52, 53). Surprisingly, one study of ulcerative colitis found MSI more frequently in patients whose colonic mucosa was negative for dysplasia (50%) than in those with cancer (40%; Ref. 53). In this context, the relative infrequency (9%) of MSI-H among our IBD neoplasms is noteworthy. We did not observe such a high prevalence of MSI in the current study. Nevertheless, it is possible that our choice of five NIH consensus microsatellite markers resulted in an underestimation of MSI prevalence, because a unique set of oligonucleotide repeat loci may be altered in each type of neoplasm (54–56).

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