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Frequent Loss of hMLH1 by Promoter Hypermethylation Leads to Microsatellite Instability in Adenomatous Polyps of Patients with a Single First-Degree Member Affected by Colon Cancer[1]

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ABSTRACT

The first-degree relatives of patients affected by colorectal cancer, who do not belong to familial adenomatous polyposis and hereditary nonpolyposis colorectal cancer families, have a doubled risk of developing tumors of the large intestine. We have previously demonstrated that subjects with a single first-degree relative (SFDR) with colon cancer have a doubled risk for developing colorectal adenomas, and in these cases, polyps recur more frequently. The mechanism underlying this predisposition has not been clarified. In this study, we evaluated the frequency of microsatellite instability (MSI) using the five markers suggested by the National Cancer Institute workshop, target gene mutations, hMLH1and hMSH2 expression, and *hMLH1* promoter hypermethylation in the adenomas of patients with and without a SFDR affected by colon cancer. Seventy polyps were obtained from 70 patients: 27 with a single FDR with colon cancer and 43 without such a history. Of the 70 polyps, 12 were MSI-H (17.1%), 20 were MSI-L (28.6%), and 30 were microsatellite stable (42.9%). Of the 27 patients with positive family history, 8 polyps (29.6%) were MSI-H compared with those with negative history in which 4 polyps (9.3%) were MSI-H ($P < 0.02$). Of the 12 MSI-H polyps, all of the polyps obtained from patients with positive family history had loss of hMLH1 immunostaining *versus* one with negative family history ($P < 0.02$). Of the MSI-H polyps, 2 had a somatic frameshift mutation of the *MBD4* gene, 1 of *MSH6*, 1 of *BAX*, and 2 of transforming growth factor βRII. Furthermore, 6 of 8 polyps from patients with positive family history with MSI-H and loss of MLH1 had hypermethylation of the *MLH1* promoter *versus* none of the MSI-H with negative family history ($P < 0.02$). All 6 polyps of the 27 from SFDR positive subjects, with *hMLH1* promoter hypermethylation loss of hMLH1 and MSI, were located in the right colon ($P < 0.02$). Hypermethylation of the promoter of hMLH1, consequent loss of hMLH1 expression, and MSI are at the basis of ~25% of adenomatous polyps developed in subjects with a SFDR affected by colorectal cancer.

INTRODUCTION

CRC[3] is the third leading cause of cancer-related deaths worldwide. Almost 70% of all CRC are sporadic. Of the remaining 30%, a small percentage belong to the familial syndromes HNPCC and FAP, whereas the vast majority show nonsyndromic familial susceptibility. In fact, first-degree relatives of patients affected by CRC, who do not fulfill the criteria for the diagnosis of FAP and HNPCC, have a doubled risk of developing tumors of the large intestine. This risk increases by 4–6-fold as the number of affected relatives increases (1). Moreover, first-degree relatives of patients with adenomas are at increased risk for CRC (2). We have previously demonstrated that subjects with a SFDR affected by colon cancer have a doubled risk of developing adenomatous polyps compared with patients without such a history, and polyps are often located on right side and show higher degree of dysplasia (3). Furthermore, in this group of patients, polyps recur more frequently over a 3-year period of follow-up after a successful polypectomy and the achievement of a colon free of polyps at colonoscopy (4).

Most CRCs arise from the preexisting adenomatous polyps through the accumulation of genetic changes that start with the loss of adenomatous polyposis coli function (5–7). Two distinct pathways of CRC progression have been defined (8). The first of these is called the CIN pathway, which occurs in >80% of colon cancers and is characterized by LOH and gross chromosomal rearrangements (9). The second involves a mutator phenotype and is characterized by MSI (10, 11). MSI is caused by mutations (12) or promoter methylation (13) of the key DNA MMR genes. Germ-line mutations of such genes is the basis of HNPCC (14, 15). CRCs evolving through the CIN or MSI pathways have distinct clinical and pathological features with MSI tumors being often right-sided, mucinous, and predominantly diploid compared with those characterized by CIN (16). Furthermore, although histologically more aggressive, MSI tumors tend to be less metastatic as well (17).

The mechanisms underlying the predisposition to develop colonic adenomas in subjects with a SFDR with CRC have not been established and are poorly understood. In this study, we sought to evaluate the rate of MSI, the expression of the MMR proteins hMLH1 and hMSH2, and the methylation status of *hMLH1* promoter, in patients with and without a SFDR affected by CRC, to elucidate the mechanistic basis of this predisposition.

MATERIALS AND METHODS

Tissue Samples and DNA Extraction. For the purpose of this study, we obtained adenomatous polyps from a pool of 172 adenomas that were resected from 70 patients between May 1990 and April 1992 at the Division of Gastroenterology and Digestive Endoscopy, Policlinico S.Orsola-Malpighi, Bologna, Italy. None of the patients had >5 polyps. In those patients with multiple polyps, only the largest adenoma was studied, and therefore 70 adenomatous polyps were considered for DNA extraction and histological evaluations. Of the 70 patients, 40 were males and 30 were females; 27 (38.6%) had SFDR with colon cancer and 43 (61.4%) had a negative history. Family history and patients' pedigrees were carefully recorded by qualified personnel and confirmed by hospital records. Patients with HNPCC, FAP, and with positive family histories for cancers other than CRC were excluded. Only

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[3] The abbreviations used are: CRC, colorectal cancer; HNPCC, hereditary nonpolyposis colorectal cancer; FAP, familial adenomatous polyposis; SFDR, single first-degree relative; CIN, chromosomal instability; MSI, microsatellite instability; MSI-L, MSI low; MSI-H, MSI high; MMR, mismatch repair; NCI, National Cancer Institute; LOH, loss of heterozygosity; TGF, transforming growth factor; MSP, methylation-specific PCR.

patients with a SFDR affected by CRC or patients without more complex family histories of cancer were included in this study.

Sections from adenomas were reviewed by a pathologist to assess pathological parameters according to published criteria and without knowledge of the status of the patient's family history.

Characteristics of polyps were classified as follows: (*a*) size: <5 mm, 6–10 mm, and >10 mm; (*b*) pathology: tubular, tubulovillous, and villous; (*c*) dysplasia: mild, moderate, and severe; and (*d*) location: left (from rectum to the splenic flexure) and right (splenic flexure to cecum). Tissues were carefully microdissected from adenomas and normal surrounding tissue (as negative control) as described previously (18). DNA extraction was performed with the phenol:chloroform procedure using standard protocol.

MSI Testing. MSI was assessed using the five markers recommended by the NCI workshop (19). MSI high adenomas were defined as those having two of the five markers unstable. Assessment of LOH was assigned when a tumor allele showed at least a 50% reduction in the relative intensity of one allele in neoplastic tissue compared with the matched normal DNA. Additionally, mutations in the coding microsatellite sequences of *BAX*, *hMSH3*, *hMSH6*, *MBD4*, and *TGF-βRII* was also undertaken. Briefly, PCR reactions were carried out in a PTC 200 thermal cycler (MJ Research, Waltham, MA) using the forward oligonucleotide dye-labeled method (Beckman dyes, Research Genetics; Invitrogen Corporation, Huntsville, AL), following published protocols and primer sequences (18, 20–22). MSI analysis was performed with a Beckman Coulter sequencer CEQ 2000 xl (Beckman Coulter).

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections were deparaffinized and rehydrated, then subjected to antigen retrieval by incubation with 0.01 M citrate buffer (pH 6.0) in a microwave oven. After cooling, slides were washed in PBS-T (PBS + 0.1% Tween 20), and endogenous peroxidase activity was blocked using 0.1% H_2O_2 in PBS-T. After washing in PBS-T, endogenous biotin was blocked using the Biotin Blocking Kit (Dako A/S, Glostrup, Denmark). The whole procedure was performed with the Catalyzed Signal Amplification System (Dako A/S), using the anti-hMLH1 antibody at a dilution of 1:200 (clone G168-15l; PharMingen, San Diego, CA) and the anti-hMSH2 antibody at the dilution of 1:50 (G219-1129; PharMingen). After development, slides were counterstained with Meyer's hematoxylin and dehydrated through ascending grades of alcohols and mounted. Normal epithelium and stromal cells provided a positive internal control. Staining of tumor cells was evaluated as present or absent in stained slides.

MSP. DNA samples were bisulfite treated following published protocols (23). MSP was performed for the region C of the *hMLH1* promoter which correlates with loss of protein expression. MSP can distinguish methylated from unmethylated alleles based on sequence alterations of DNA produced by bisulfite treatment. Briefly, 500 ng to 2 μg of total genomic DNA were denatured with NaOH and modified by sodium bisulfite, followed by purification with Wizard DNA purification resin (Promega Corporation, Madison, WI), treated with NaOH, precipitated with ethanol, and sodium acetate, and finally resuspended in water.

Three μl of solution containing purified DNA were used as template for PCR reactions for a total volume of 25 μl, including 10× PCR buffer (16.6 mM ammonium sulfate, 67 mM Tris (pH 8.8), 6.7 mM $MgCl_2$, and 10 mM 2-mercaptoethanol), 200 μM each deoxynucleotide triphosphates, and 25 pmol of each primer. After denaturation at 95°C for 10 min, 1 unit of Taq DNA polymerase (Invitrogen Corporation, Carlsbad, CA) was added to each sample. PCR reactions were carried out in a PTC 200 thermal cycler (MJ Research) for 40 cycles (30s at 95°C, 30 s at 65°C for the methylated and 30 s at 60°C for the unmethylated allele, 30 s at 70°C and a final extension at 72°C for 7 min). Water and bisulfite-treated DNA extracted from the cell line SW48 were used as negative and positive controls for each reaction. Ten μl of PCR product were run in a 4% Nu-sieve GTG-agarose gel in Tris-borate EDTA buffer and visualized after ethidium bromide staining.

Statistical Analysis. Comparisons of variables between groups were analyzed by univariate statistics. To establish the statistical significance of observed differences, the χ^2 test and the Fisher's exact test for dichotomous variables were used when appropriate. Ps < 0.05 were considered statistically significant. Data are expressed as percentages (Table 2). Moreover, to verify homogeneity for demographic variables, the Kolmogorov-Smirnov's test and the Student's t test for continuous variables were calculated. Ps < 0.05 were considered statistically significant. Data are expressed as means ± SD (Table 1).

All statistical analyses were done with the SPSS/PC (SPSS Inc., Chicago, IL) statistical package.

RESULTS

Polyps' Features. We analyzed 70 polyps obtained from 70 patients. Of these patients, 27 had a SFDR affected by CRC, whereas 43 had a negative family history for the disease. Characteristics of patients as well as of polyps are described in Table 1. Of the 70 polyps, 23 were tubular, whereas 47 (32.9 *versus* 67.1%) had villous features; meanwhile, 48 were located in the left colon, and 22 were on the right (68.6 *versus* 31.4%). Eleven (15.7%) had mild, 38 (54.3%) had moderate, and 21 (30%) had severe dysplasia. Among the 27 patients with a SFDR, 15 polyps were located in the right colon (55.5%) and 12 in the left, whereas in the 43 patients without a SFDR 7 polyps were from the right (16.3%) and 36 from the left colon.

MSI Status in Adenomatous Polyps. We performed the MSI analysis using the five markers recommended by the NCI workshop (Ref. 19; Fig. 1). The frequency of MSI and LOH was as follows: 12 of 70 (17.1%) polyps had MSI-H; 20 of 70 (28.6%) were MSI-L; 30 (42.9%) were MSS; and 8 of 70 (11.4%) had LOH. Of the 27 patients with a positive family history, 8 (29.6%) had polyps with MSI-H, whereas in the 43 patients with a negative family history, 4 polyps (9.3%) were MSI-H ($P < 0.05$). On the other hand, 23 of 43 polyps (53.5%) from patients with a negative family history were MSS, whereas 7 of 27 (25.9%) from patients with a positive family history were MSS ($P < 0.03$). No differences between the two groups were observed regarding MSI-L and LOH. Seven of 22 polyps (31.8%) located in the right colon were MSI-H when compared with 5 of 48 polyps (10.4%) located in the left colon ($P < 0.04$; Table 2). Interestingly all of the 7 polyps with MSI-H located in the right colon were from SFDR patients, whereas of the 5 located in the left, 1 was from a SFDR patient and 4 from patients without such a history. There was no statistical difference between MSI status compared with histology, sex, and age.

We then determined the frequency of mutations of the five markers used for the MSI analysis. BAT 25 and BAT 26 were mutated in 18.6% (13 of 70) and 8.6% (6 of 70) of cases, respectively. *D2S123* had LOH in 5.7% of cases (4 of 70) and MSI in 18.6% of cases (13 of 70). *D5S346* showed LOH in 4.3% of cases (3 of 70) and MSI in 21.4% of cases (15 of 70), whereas *D17S250* had LOH 2.9% of cases (2 of 70) and MSI in 7.1% of cases (5 of 70). Thus, *D5S346*, BAT25, and *D2S123* were more frequently mutated than BAT26 and *D17S250* (Fig. 2).

Frameshift Mutations of Target Genes. We analyzed by PCR amplification the frameshift mutations of the region encompassing the

Table 1 *Patients and polyps features*

Patients		Polyps			Family history	
Sex	Age avg. (range 41–84 yr)	Histology	Polyp location	Dysplasia	Positive/negative	Polyp location
40 males (57.1%)	63.6 ± 9.53 yr[a]	23 tubular (32.9%)	48 left side (68.6%)	11 mild (15.7%)	27 positive (38.6%)	15 right side (55.5%)
30 females (42.9%)	61.9 ± 8.62 yr[a]	47 villous features (67.1%)	22 right side (31.4%)	38 mod. (54.3%)	43 negative (61.4%)	7 right side (16.3%)
				21 sev. (30%)		

[a] $P > 0.05$.

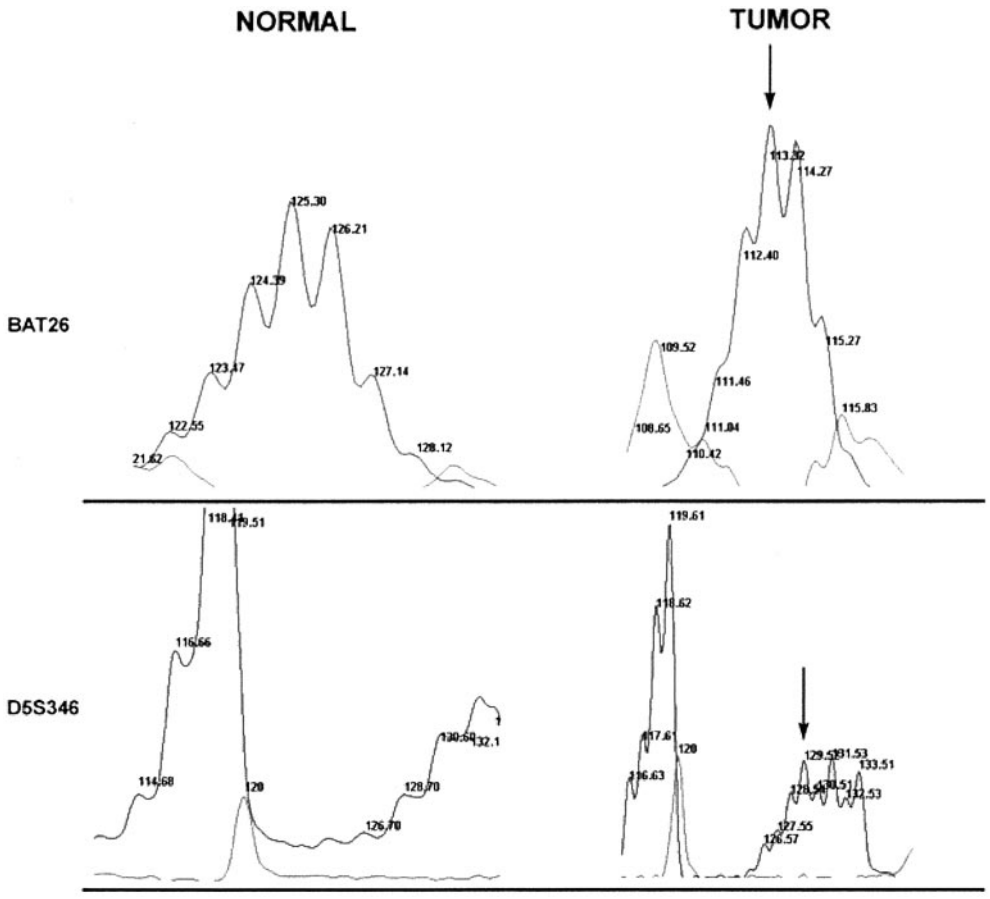

Fig. 1. MSI shown at BAT26 and *D5S346* of a patient with loss of MLH1 attributable to promoter hypermethylation. For each microsatellite marker, DNA extracted from normal colonic epithelium was used as negative control.

polynucleotide tracts of the *TGF-β RII*, *hMSH3*, *hMSH6*, *BAX*, and *MBD4*. Of the polyps with MSI-H, 2 had mutations at *MBD4* gene (1 with positive and 1 with negative family history), 1 at *hMSH6* (with positive family history), 2 at *TGF-βRII* (with positive family history), and 1 at *BAX* genes (with positive family history). Five of these polyps had severe dysplasia and were collected from patients with a positive family history. None of the MSI-L, MSS, or LOH polyps had mutations at these target genes.

Expression of hMLH1 and hMSH2. We then performed immunohistochemistry for hMLH1 and hMSH2 proteins. Regarding hMLH1 staining, 9 polyps showed loss of expression of the protein *versus* 61 with normal expression (12.9 *versus* 87.1%). All these polyps were MSI-H. Of these, 8 were from patients with positive family history *versus* 1 with a negative history ($P < 0.02$; Fig. 2). None of the MSI-L, MSS, or LOH polyps had loss of hMLH1 protein. All polyps had normal expression of the hMSH2 protein.

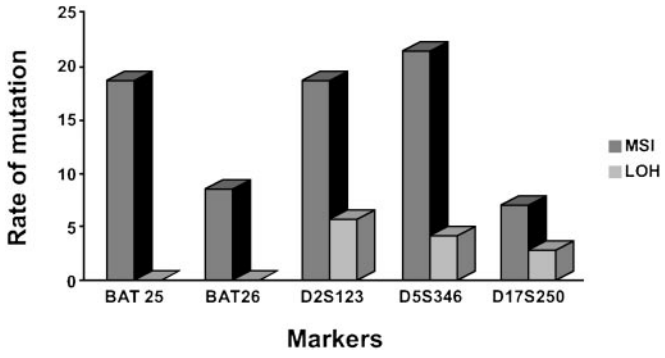

Fig. 2. Frequency of MSI and LOH at each of the markers used in the study. BAT25, D5S346, and D2S123 were the most frequently mutated.

Table 2 *The frequency of MSI in adenomatous polyps*

	LOH	MSS	MSI-L	MSI-H
Overall MSI	11.4%	42.9%	28.6%	17.1%
Family history				
Positive ($n = 27$)	18.6% (5)	25.9% (7)[a]	25.9% (7)	29.6% (8)[b]
Negative ($n = 43$)	7% (3)	53.5% (23)[a]	30.2% (13)	9.3% (4)[b]
Polyp location				
Right side ($n = 22$)	13.6% (3)	36.4% (8)	18.2% (4)	31.8% (7)[c]
Left side ($n = 48$)	10.4% (5)	45.7% (22)	33.3% (16)	10.4% (5)[c]
Target gene mutations	0	0	0	6/12
Positive family history				5/6
Loss of MLH1 exp.	0	0	0	9/12
Positive family history				8/9[d]
MLH1 prom. meth.	0	0	0	6/9
Positive family history				6/6[e]
Right side location				6/6[f]

[a] $P < 0.03$.
[b] $P < 0.05$.
[c] $P < 0.04$.
[d] $P < 0.02$.
[e] $P < 0.02$.
[f] $P < 0.02$.

hMLH1 Promoter Hypermethylation. Finally, we addressed whether loss of hMLH1 protein was because of promoter hypermethylation. To address this, we performed MSP after bisulfite treatment of DNA (Fig. 3). Six of 8 polyps showing loss of hMLH1 staining in patients with a positive family history also had *hMLH1* promoter hypermethylation, whereas none of the MSI-H polyps from patients with a negative history had this result ($P < 0.02$). All six polyps with *hMLH1* promoter hypermethylation were located in the right colon ($P < 0.02$). None of the MSI-L, MSS, or LOH polyps had *hMLH1* promoter hypermethylation (Fig. 4).

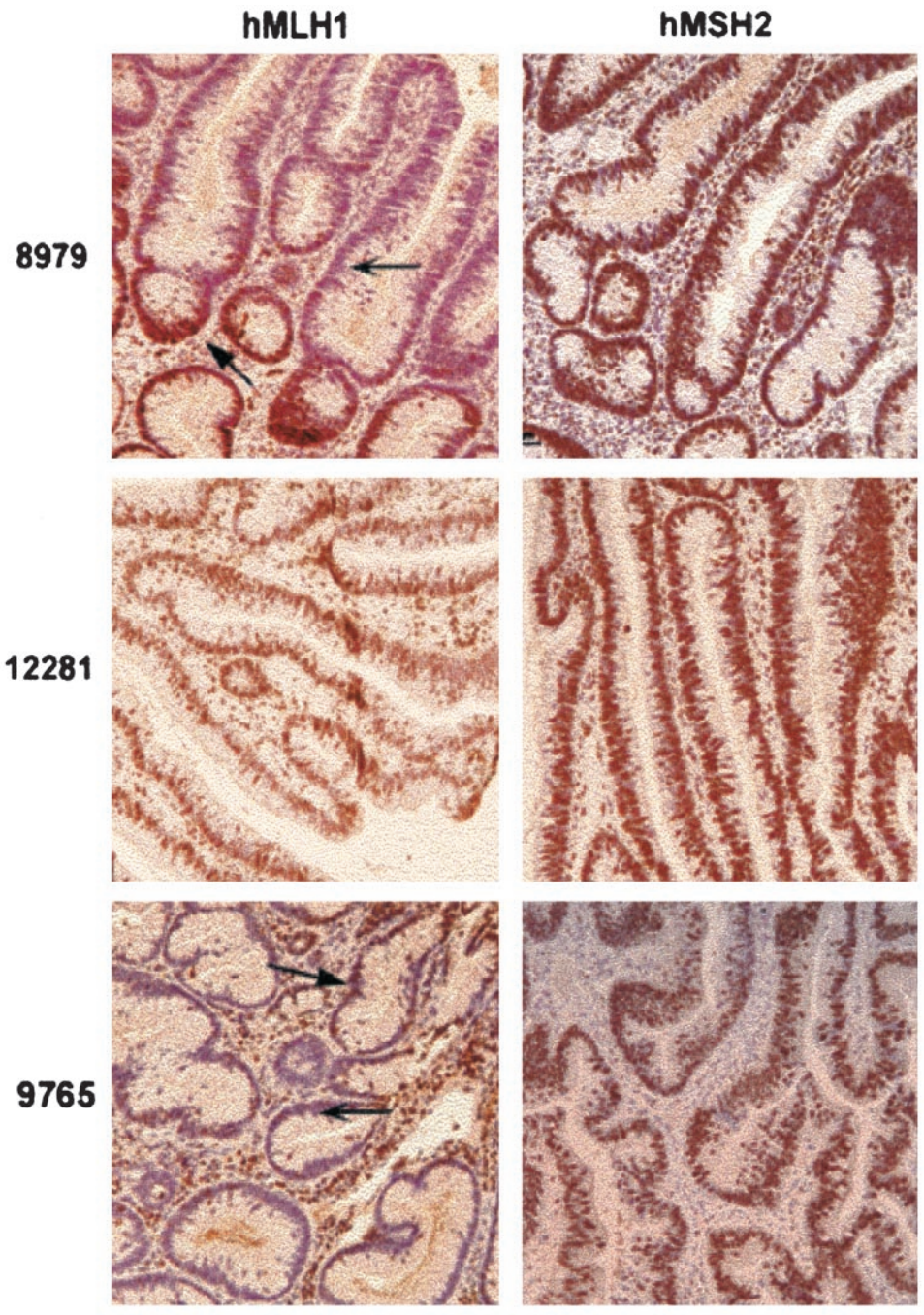

Fig. 3. Protein expression of hMLH1and hMSH2 (*left and right panels*, respectively) in patients with MSI-H and positive family history (8979 and 9765) and with MSS polyp (12281). None of the polyps had loss of hMSH2.

DISCUSSION

In this study, we analyzed defective DNA MMR as a possible mechanism that could explain the predisposition of SFDR of patients with CRC to develop adenomatous polyps of the colon. We found that of 27 patients with a positive family history, ~30% had MSI, compared with 9.3% (4 of 43) in those without such a history. We also showed that in ~25% of cases (6 of 27), this phenomenon is because of loss of hMLH1 expression attributable to promoter hypermethylation. Furthermore, MSI polyps with loss of hMLH1 are located proximally to the splenic flexure and have frequent severe dysplasia, compared with MSS polyps. The overall high rate of MSI (17.1%) found in our study might be explained by the number of those having a SFDR affected by colon cancer. In 6 cases, polyps carried mutations at target genes: 2 of them involved *TGF-βRII*; 1 at *BAX*; 2 at *MBD4*; and 1 at *MSH6*. Five of these polyps from patients with positive family history had severe dysplasia and suggesting that target gene mutations are linked to switch to malignancy (24).

While a number of investigations have recently been published on the role of MSI in the process of colorectal carcinogenesis, the data

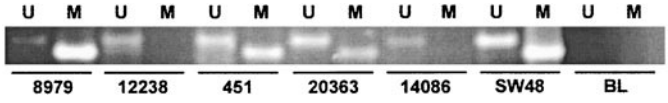

Fig. 4. MSP for *hMLH1* promoter. In this panel, three polyps with positive family history, MSI-H, and loss of MLH1 had promoter hypermethylation (8979, 451, 20363). Two polyps with MSI-L and with normal protein staining had no methylation. SW48 was used as a positive control, and water (*BL*) was used as negative. All samples were run with both PCR products for unmethylated (*U*) and methylated (*M*) portions.

focused upon colorectal adenomas are difficult to compare. There is a wide range of MSI reported in previous studies that ranges from 3 to 32% for MSI-L and 1–7% for MSI-H in sporadic adenomas and majority of these being MSI-L adenomas (24–28). The major factor determining this discrepancy is the microsatellite markers used in these studies and the criteria used to define varying degrees of MSI. Only half of the studies distinguished between MSI-H and MSI-L, and moreover, none of the studies used the guidelines recommended by the NCI workshop to study MSI (19). Additionally, none of the previous studies has evaluated the role of MSI in colorectal adenomas from patients with a positive family history for CRC but not involved in FAP or HNPCC. The adopted criteria of our study were to use the standard markers suggested by the NCI workshop and widely accepted for the screening of MSI in colonic tumors that has not been reported before for adenomatous polyps. This also might explain the high sensitivity of the panel of these five markers in detecting MSI tumors. In previous studies, the low rates of MSI found in polyps or aberrant crypt foci, which are the precursors of adenomas, were described by authors using markers such as BAT 26 or BAT 40 (26, 29–31) that have a high sensitivity for cancers but low for polyps or markers different from those proposed by the NCI workshop. Interestingly, in our study we found that *D5S346* and BAT 25 markers were most frequently mutated, whereas BAT 26 was less frequently mutated, thus confirming the previously reported data that BAT 26 has a low sensitivity in detecting MSI in adenomas.

MSI in HNPCC patients occurs as a result of germ-line mutations at genes such as *hMLH1*, *hMSH2*, *hMSH6*, and *hMLH3* (12, 15, 32–34) that comprise the DNA MMR system, whereas in sporadic cancers, at least 90% of cases with MSI are attributable to hypermethylation of the promoter of *hMLH1* (35–37). This phenomenon of epigenetic silencing of tumor suppressor gene function occurs at clusters of C-G rich sequences of the gene promoters and has been defined as CpG island mutator phenotype (38–40). CpG island mutator phenotype-positive tumors have a high degree of CpG island methylation and a high frequency of classical genetic changes such as MSI and *TGF-β RII* mutations (38). Recently, Miyakura *et al.* (36) reported that ~90% of sporadic cancers with MSI have hMLH1 promoter's hypermethylation. Those with full methylation pattern of the promoter were exclusively located in the proximal colon. It would have been of interest to stratify that population of patients by simple family history.

The fact that MSI is evident in adenomas and that MSI and methylation are observed simultaneously suggests that MSI and hypermethylation are dependent on each other. The underlying causes of promoter hypermethylation are not fully understood.

It has been suggested that perturbation of methylation might be produced through the diet and cigarette smoking (41, 42). In our study, the age of patients with a SFDR with colon cancer, who developed MSI-H adenomas, was relatively higher than HNPCC patients. This suggests that familial MSI-H adenomas attributable to *hMLH1* promoter methylation might be the result of an environmental or a combined genetic-environmental predisposition.

Given our data, the analysis of MSI in adenomatous polyps of patients with positive family history of CRC is useful in assessing the true risk of developing cancer. Thus, patients with a SFDR with colon cancer should undergo total colonoscopy (3, 43), and if adenomas are found, MSI testing should be performed to assess cancer risk.

In conclusion, our study shows for the first time that *hMLH1* promoter hypermethylation and MSI are early events in colon carcinogenesis and are the basis of the high predisposition to develop adenomas and, eventually cancer, in ~25% of subjects with a SFDR affected by CRC.

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