

The Frequency of Hereditary Defective Mismatch Repair in a Prospective Series of Unselected Colorectal Carcinomas

Julie M. Cunningham,¹ Cheong-Yong Kim,^{1,*} Eric R. Christensen,¹ David J. Tester,¹ Yann Parc,^{1,#} Lawrence J. Burgart,¹ Kevin C. Halling,¹ Shannon K. McDonnell,² Daniel J. Schaid,² Catherine Walsh Vockley,³ Vickie Kubly,¹ Heidi Nelson,⁴ Virginia V. Michels,³ and Stephen N. Thibodeau¹

Departments of ¹Laboratory Medicine and Pathology, ²Health Sciences Research, ³Medical Genetics, and ⁴Surgery, Mayo Clinic, Rochester, MN

A comprehensive analysis of somatic and germline mutations related to DNA mismatch–repair (MMR) genes can clarify the prevalence and mechanism of inactivation in colorectal carcinoma (CRC). In the present study, 257 unselected patients referred for CRC resection were examined for evidence of defective DNA MMR. In particular, we sought to determine the frequency of hereditary defects in DNA MMR in this cohort of patients. MMR status was assessed by testing of tumors for the presence or absence of hMLH1, hMSH2, and hMSH6 protein expression and for microsatellite instability (MSI). Of the 257 patients, 51 (20%) had evidence of defective MMR, demonstrating high levels of MSI (MSI-H) and an absence of either hMLH1 ($n = 48$) or hMSH2 ($n = 3$). All three patients lacking hMSH2, as well as one patient lacking hMLH1, also demonstrated an absence of hMSH6. DNA sequence analysis of the 51 patients with defective MMR revealed seven germline mutations—four in *hMLH1* (two truncating and two missense) and three in *hMSH2* (all truncating). A detailed family history was available for 225 of the 257 patients. Of the seven patients with germline mutations, only three had family histories consistent with hereditary nonpolyposis colorectal cancer. Of the remaining patients who had tumors with defective MMR, eight had somatic mutations in *hMLH1*. In addition, hypermethylation of the hMLH1 gene promoter was present in 37 (88%) of the 42 hMLH1-negative cases available for study and in all MSI-H tumors that showed loss of hMLH1 expression but no detectable *hMLH1* mutations. Our results suggest that, although defective DNA MMR occurs in ~20% of unselected patients presenting for CRC resection, hereditary CRC due to mutations in the MMR pathway account for only a small proportion of patients. Of the 257 patients, only 5 (1.9%) appear to have unequivocal evidence of hereditary defects in MMR. The epigenetic (nonhereditary) mechanism of *hMLH1* promoter hypermethylation appears to be responsible for the majority of the remaining patients whose tumors are characterized by defective DNA MMR.

Introduction

Familial polyposis (FAP [MIM 175100]) and hereditary nonpolyposis colorectal cancer (HNPCC [MIM 114500]) are two common autosomal dominant disorders predisposing to the development of colorectal cancer (CRC) (Bellacosa et al. 1996; Khine et al. 1996; Fante et al. 1997; Lynch et al. 1997; Soravia et al. 1997). FAP is characterized by the presence of hundreds to thousands of adenomatous polyps. HNPCC, on the other hand, is characterized by the occurrence of colorectal cancer at an early age (fourth to sixth decade),

a tendency to develop multiple primary cancers, and an increased risk for the development of certain other cancers, particularly endometrial and stomach cancer (Lynch and Lynch 1995). It is generally easier to ascertain a diagnosis of FAP than HNPCC, because most patients with FAP eventually develop profuse polyposis and, sometimes, other tumor types (e.g., osteomas and periampullary carcinomas) that can aid in the diagnosis. Patients with HNPCC, on the other hand, lack distinctive phenotypic features. Consequently, the diagnosis of HNPCC has historically been based on family history. As a result, the true incidence of this disease and the underlying molecular defects have been difficult to establish. The estimated percentage of CRC cases caused by HNPCC has varied from ~0.5% to 10% (Aaltonen et al. 1994b; Bellacosa et al. 1996; Brassett et al. 1996; Evans et al. 1997; Peel et al. 2000).

The Amsterdam criteria (AC) were established in the early 1990s to define the clinical criteria used to identify patients with HNPCC (Vasen et al. 1991). These criteria

Received June 11, 2001; accepted for publication August 7, 2001; electronically published August 24, 2001.

Address for correspondence and reprints: Dr. Stephen N. Thibodeau, Laboratory Genetics/HI 970, Mayo Clinic, 200 First Street SW, Rochester, MN 55905. E-mail: sthibodeau@mayo.edu

* Present affiliation: Chosun University Hospital, Kwangju, Korea.

Present affiliation: Centre de Chirurgie Digestive, Paris.

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0002-9297/2001/6904-0012\$02.00

call for the occurrence of verifiable colorectal cancer in three individuals (one of whom is the first-degree relative of the other two), the presence of cancer in at least two successive generations, and an age at onset of CRC of <50 years in at least one case. These criteria were created to help identify families that have a high probability of having an hereditary form of colorectal cancer not accompanied by polyposis (i.e., HNPCC). It was understood, however, that families classified, on the basis of the AC, as having HNPCC might represent a heterogeneous group with respect to the underlying basis of disease. Nonetheless, it was an important step in attempting to determine the genetic basis of colorectal cancer.

For clinical purposes, however, the AC are now recognized to be too restrictive, since other malignancies (e.g., gastric and endometrial) frequently occur in patients with HNPCC and may be the presenting cancers in such families (Beck et al. 1997b; Bapat et al. 1999). As a result, several modifications that include consideration of extracolonic malignancies in the diagnosis have been suggested (Beck et al. 1997b; Bapat et al. 1999; Vasen et al. 1999). In addition to family history, however, there are now laboratory approaches that may aid in establishing this diagnosis in a subset of patients with HNPCC. These have arisen from studies that revealed the underlying genetic defect in many—but not all—families diagnosed with HNPCC; namely, defective DNA-mismatch repair (MMR).

Of the seven human DNA MMR genes that have been identified (*bMLH1*, *bMSH2*, *bMSH3*, *bMSH6*, *bPMS1*, *bMLH3*, and *bPMS2*), germline mutations have been identified in all but two (*bMSH3* and *bMLH3*) in families with HNPCC (Fishel et al. 1993; Lindblom et al. 1993; Papadopoulos et al. 1994, 1995; Lipkin et al. 2001). Germline mutations are detected most frequently in patients with HNPCC who satisfy the AC, although some individuals who do not fulfill these criteria also carry germline mutations (Beck et al. 1997a). The majority of these HNPCC patients have mutations in either *bMLH1* or *bMSH2*, with less frequent involvement of other genes. However, there are also some families with HNPCC in which mutations in MMR genes have not been identified and whose tumors do not demonstrate phenotypic evidence of defective DNA MMR—that is, they do not demonstrate either the presence of tumor microsatellite instability (MSI) or the absence of protein expression for one of the genes involved in DNA MMR (Aaltonen et al. 1994a; Craanen et al. 1996; Moslein et al. 1996; Lamberti et al. 1999; Peel et al. 2000).

MSI is a phenotypic indicator of defective DNA MMR and is detected in tumors from HNPCC patients, as well as in a low percentage of various sporadic cancers, such as colon, endometrial, and stomach cancer (Aaltonen et al. 1993; Han et al. 1993; Ionov et al.

1993; Thibodeau et al. 1993; Burks et al. 1994; Duggan et al. 1994; Keller et al. 1995; Akiyama et al. 1996; Caduff et al. 1996; Battista et al. 1997; Gurin et al. 1999). In CRC, several phenotypes of MSI have been defined: MSI-H (MSI in >30% of loci examined), MSI-L (MSI in <30% loci examined), and MSS (microsatellite stable, showing no instability at any site) (Boland et al. 1998). Sporadic CRC with the MSI-H phenotype has distinct clinicopathological features, including a tendency to occur in the proximal colon, high frequency in females, high grade, diploidy, and improved overall survival compared with those without widespread microsatellite instability (Thibodeau et al. 1993; Fujita et al. 1996; Sankila et al. 1996; Lynch et al. 1997). Almost all such cases are due to functional loss of either *bMLH1* or *bMSH2*. The MSI-L phenotype, on the other hand, is not associated with these clinicopathological features or with altered *bMLH1* or *bMSH2*. The majority of HNPCC-associated tumors are characterized by MSI-H, whereas MSI-H, MSI-L, and MSS are found in sporadic CRC.

In spite of numerous studies on the frequency of defective MMR in CRC, there have been few studies examining the frequency of inherited CRC due to defective MMR in a series of patients not selected for family history of colon cancer. Aaltonen et al. (1998), in a study of 509 consecutive Finnish patients with colorectal cancer, found that inherited defects in MMR occurred in ~2% of the cases studied. In their study, all cases were examined for evidence of defective MMR, irrespective of family history; however, half (5/10) of the germline mutations identified were determined to originate from a single common founder mutation, found in >30 families in Finland and Sweden. No other population-based studies have been performed. The purpose of the present study was to determine the frequency of defective DNA MMR, as well as the underlying mechanism, in an unselected prospective series of colorectal cancers in patients referred to the Mayo Clinic. More specifically, we sought to determine the frequency of inherited defects in this series of patients. The present article details the first set of 257 unselected cancers in an ongoing prospective study. Tumors were examined for the presence or absence of: (a) tumor MSI; (b) hMLH1, hMSH2, and hMSH6 protein expression; (c) *bMLH1*, *bMSH2*, and *bMSH6* gene mutations; and (d) *bMLH1* and *bMSH2* promoter hypermethylation. Patients' family histories were obtained to determine the familial component, and the clinical characteristics of the patients were analyzed.

Material and Methods

Patient Population

A total of 514 patients underwent surgical resection for CRC at the Mayo Clinic during a 1.5-year period,

from December, 1995, to April, 1997. Four hundred and sixty-six patients were approached about the study, and 267 (57.3%) consented to participate. Of the consenting patients, seven had inadequate cancer tissue available for study, one had only paraffin-embedded tissue available, and two had only metastatic disease. These patients were not included, leaving 257 available for further study. Of the 257 patients, 237 were white (92%), 4 (1.5%) were of African American descent, 3 (1%) were of Hispanic origin, and 13 (5%) had no designated race. Seventy-five percent (192) came from Minnesota (103), Iowa (32), Wisconsin (10), North Dakota (6), South Dakota (7), or Illinois (34). The remainder came from an additional 27 states within the continental United States.

Patient chart reviews were performed to obtain clinical characteristics of the tumor, including tumor site, stage, and age at diagnosis. For tumor site, tumors of the proximal colon were defined as those CRCs occurring in the cecum, the ascending colon, or the transverse colon. Distal tumors were defined as those occurring in the descending or sigmoid colon or in the rectum. Family histories were documented by telephone follow-up for 225 of the 257 patients, through use of a detailed questionnaire. On the basis of this information, three- to four-generation pedigrees were constructed. On the basis of the presence of family history of CRC, patients fulfilling the AC ($n = 7$) (Vasen et al. 1991) and patients fulfilling the modified AC ($n = 9$, including the seven AC) (Vasen et al. 1999) were identified. For the purposes of the present study, an HNPCC-related cancer included cancers of the colon, small bowel, ureter, endometrium, and renal pelvis (Vasen et al. 1999). FAP was diagnosed in three patients, and chronic ulcerative colitis (CUC) was diagnosed in another four; these patients were not excluded from the analysis.

DNA Extraction

DNA was extracted from frozen or paraffin-embedded tissues, as described elsewhere (Thibodeau et al. 1998). In brief, DNA from microdissected frozen tissue sections (10 μm) was extracted by a standard phenol/chloroform procedure. For tumor DNA, only those areas containing >70% tumor cells were used. For DNA extraction from paraffin-embedded tissues, the Qiamp Tissue kit (Qiagen, Inc.) was used, according to the manufacturer's instructions. The corresponding normal control DNA for each patient was derived from peripheral blood. For these specimens, DNA was extracted using the Puregene nucleic acid-isolation kit (Gentra).

Microsatellite Instability

Paired normal and tumor DNA were analyzed for microsatellite instability with six dinucleotide microsatellite markers (D5S346, TP53, D18S34, D18S49,

D18S61, and ACTC) and one mononucleotide repeat (BAT 26). PCR and gel electrophoresis were performed as described by Thibodeau et al. (1993). Tumors were classified as MSH-H if $\geq 30\%$ markers demonstrated instability, as MSH-L if $< 30\%$ demonstrated MSI, and as MSS if no marker exhibited MSI (Boland et al. 1998; Thibodeau et al. 1998). These studies were performed with DNA isolated from paraffin-embedded material.

Immunohistochemical Analysis

The expression of hMLH1, hMSH2, and hMSH6 protein was assessed as described elsewhere (Thibodeau et al. 1998). In brief, 5- μm tissue sections from formalin-fixed, paraffin-embedded tissue were stained with antibody to hMLH1 (clone G168 728; 1 mg/ml [PharMingen]), hMSH2 (clone FE11; 0.5 mg/ml [Oncogene Science]), and hMSH6 (clone 44, 0.5 $\mu\text{g}/\text{ml}$ [Transduction Laboratories]). Tumor cells that showed an absence of nuclear staining in the presence of normal positive staining in surrounding cells were interpreted as having an absence of expression of these proteins.

Sequencing

Exons 1-19 of *hMLH1* and/or exons 1-16 of *hMSH2* were sequenced in all tumors lacking expression of hMLH1 ($n = 48$) or hMSH2 ($n = 3$), using the ThermoSequenase kit (Amersham), essentially as described by Moslein et al. (1996). For *hMSH6*, only exon 5 was sequenced (Parc et al. 2000), since this exon contains repeat sequences commonly altered in tumors with defective DNA MMR (Yin et al. 1997; Iino et al. 2000). These studies were performed with DNA isolated from both leukocytes and fresh frozen tumor (when available).

Methylation Analysis

The methylation status of the promoter regions of both *hMLH1* and *hMSH2* was assessed as described elsewhere (Cunningham et al. 1998), using an *HpaII*-based PCR assay. As a control for the PCR, a *calcitonin* sequence devoid of *HpaII* sites was used. The *hMLH1* and *hMSH2* primers amplified fragments containing *HpaII*-sensitive sites. DNA was first digested with the restriction enzyme *HpaII*. The digested DNA was then subjected to multiplex PCR; the products were electrophoresed on 8% polyacrylamide gels and then were analyzed for the relative amplification of *hMLH1* or *hMSH2* and *calcitonin*. The sizes of the unmethylated PCR products were 114 bp, 107 bp, and 145 bp, for *hMLH1*, *hMSH2*, and *calcitonin*, respectively. Ratios of either *hMLH1* or *hMSH2* to *calcitonin* of $< .2$ indicated no methylation (as in all the normal samples tested), whereas a ratio of $> .8$ was scored as positive for hypermethylation. Some samples had intermediate values

and were considered to have either partial methylation or hemimethylation.

Some of the samples were also examined using the methylation-specific PCR (MSP) assay, as described by Herman et al. (1998). These samples were also subjected to DNA sequence analysis, after bisulfite treatment. For the sequence analysis, the following primers for PCR amplification and sequencing were designed to amplify both methylated and unmethylated modified DNA, corresponding to the regions assessed in the *HpaII* (region 3) and MSP (region 2): using the U83845 sequence, region 3, forward 5' position 339, tagattaggtatagggtttta and reverse 5' position 603, aaatataccaataaaaaaca; region 2, forward 5' position 158, gaggtttgtagtagtttt and reverse 5' position 381, ataaacacrttatttat (r = a or g, y = c or t). The underlined nucleotides represent those cytosine residues that are converted to uracil/thymine (if unmethylated) or are retained (if methylated) in the modification. The PCRs were performed in 25-μl reactions containing PCR buffer 1 (Perkin Elmer) with 1.5 mM (region 3) or 2 mM (region 2) MgCl₂, 10 pmol primer, 1.25 units *AmpliTaq* Gold (Perkin Elmer), and 1 μl bisulfite-modified DNA (equivalent to 50 ng genomic DNA). After an initial preheating step of 12 min at 95°C, PCR was performed over 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 30 min. PCR products were cleaned using a HighPure PCR purification column (Boeringer Mannheim) and were sequenced with the forward primer used in the initial PCR, on an ABI 377 (ABI Biosystems). Methylation studies were performed with DNA isolated from both leukocytes and fresh frozen tumor (when available).

Results

Patient Sample

The clinical characteristics of the 257 participating patients are shown in table 1. Patient ages at diagnosis

were 29–91 years (median 69 years), and the male-to-female ratio was 1.47 (153 male, 104 female). For the nonparticipants (n = 199), the male-to-female ratio was 1.1 (102 male, 97 female; P = .08), indicating that males were more likely to participate than females. The nonparticipants were also slightly older than were the participants (median age 73 vs. 69 years; P = .0001).

MSI and Immunohistochemistry

Of the 257 patients with CRC available for study, 51 (20%) were MSI-H, 18 (7%) were MSI-L, and 188 (73%) were MSS (table 1, fig. 1). All but one of the MSI-H tumors and none of the MSI-L or MSS tumors demonstrated MSI at BAT 26. Consistent with previous observations, significant associations were observed between tumors with the MSI-H phenotype and proximal tumor location, female gender, and Dukes' stage (table 1).

Protein expression for hMLH1, hMSH2, and hMSH6 was then examined in paraffin-embedded tissues (immunohistochemistry not shown; results summarized in fig. 1). All 51 MSI-H tumors showed an absence of protein expression of either hMLH1 (n = 48 [94%]) or hMSH2 (n = 3 [6%]). None of the tumors demonstrated an absence of hMSH6 only; however, all three tumors lacking hMSH2, as well as one with an absence of hMLH1, also showed an absence of hMSH6. All of the MSI-L and MSS tumors demonstrated normal expression for all three of these proteins (two MSS tumors were not scored for hMLH1, because of technical failure, but had normal expression of hMSH2 and hMSH6). All of the patients with FAP and CUC had normal protein expression; one patient with FAP exhibited an MSI-L phenotype, and the remaining patients exhibited an MSS phenotype.

Mechanism of Gene Inactivation

DNA sequence analysis and promoter hypermethylation studies were used to explore the mechanisms un-

Table 1

Patient Characteristics

SAMPLE (NO. AND % OF TOTAL SAMPLE)	MEDIAN AGE (RANGE) ^a (years)	NO. (%) OF INDIVIDUALS									
		Sex ^b		Tumor Site ^c		Dukes Stage ^d				Age at Diagnosis ^e	
		Male	Female	Proximal	Distal	A	B	C	D	≤50 years	>50 years
All Patients (N = 257)	69 (29–91)	153 (59.0)	104 (41.0)	120 (46.7)	137 (53.3)	16 (6.2)	112 (43.6)	86 (33.5)	42 (16.7)	24 (9.3)	233 (90.7)
MSI-H (n = 51, 19.8%)	72 (35–91)	23 (45.1)	28 (54.9)	44 (86.3)	7 (13.7)	6 (11.8)	31 (60.8)	11 (21.6)	3 (5.9)	3 (5.9)	48 (94.1)
MSI-L (n = 18, 7.0%)	65.5 (29–84)	13 (72.2)	5 (27.8)	8 (44.4)	10 (55.6)	0 (0.0)	9 (50.0)	7 (38.9)	2 (11.1)	3 (16.7)	15 (83.3)
MSS (n = 188, 73.2%)	67 (34–90)	117 (62.2)	71 (37.8)	68 (36.2)	120 (63.8)	10 (5.3)	72 (38.3)	68 (36.2)	38 (20.2)	18 (9.6)	170 (90.4)

^a P = .02 (Kruskal-Wallis test).
^b P = .05 (χ² test).
^c P = .001 (χ² test).
^d P = .01 (χ² test). Dukes Stage A—invasion limited to submucosa, with no nodal metastasis; B—invasion to at least the level of muscularis propria, with no nodal metastasis; C—invasion at any level, with regional nodal metastasis but no distant metastasis; D—distant metastasis present (Astler and Coller 1954).
^e P = .39 (χ² test).

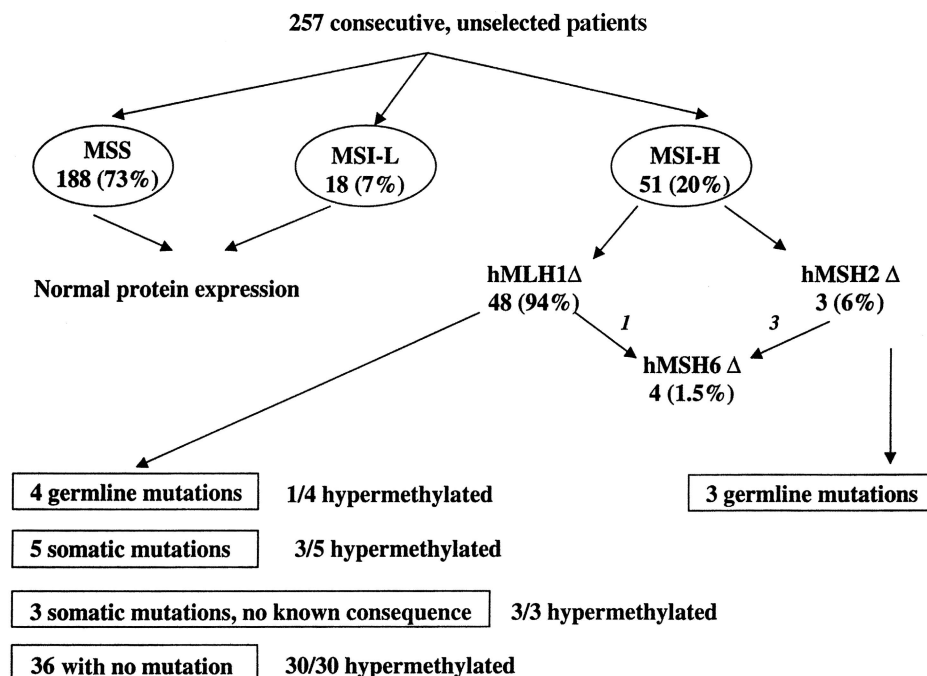


Figure 1 Flow diagram for the results of testing of the 257 consecutive, unselected patients with CRC. Tumors were assessed for the presence or absence of tumor MSI (MSI-H, MSI-L, or MSS); hMLH1, hMSH2, and hMSH6 protein expression (Δ indicates loss of expression); *bMLH1*, *bMSH2*, and *bMSH6* gene mutations; and *bMLH1* and *bMSH2* promoter hypermethylation.

derlying defective DNA MMR in the MSI-H patients ($n = 51$). Normal leukocyte DNA was available for study from all 51 MSI-H patients; however, DNA from fresh frozen tumor was available for study from only 44 of the 51 MSI-H patients (42 of 48 lacked hMLH1, and 2 of 3 lacked hMSH2). Thus, all MSI-H patients were examined for germline mutations, but only 44 of the 51 patients were examined for somatic alterations (tumor DNA sequencing and promoter methylation). The single MSI-H patient that did not show instability at BAT-26 was not examined for somatic alterations, since no frozen tissue was available for DNA extraction (this patient did not show evidence of any germline alteration in *bMLH1*).

Of the 48 patients lacking hMLH1 protein expression, 12 had *bMLH1* gene alterations, 4 of whom had germline alterations and 8 of whom had somatic alterations only (table 2, fig. 1). Two of the four germline *bMLH1* mutations were splicing alterations, which are likely to be pathogenic, whereas the other two were missense changes, which have unknown pathogenic consequences (table 2). One of these missense mutations (in patient 209) occurred at a conserved codon and might therefore have pathological consequences, whereas the other mutation (in patient 83) occurred at a nonconserved site. Two of the patients with germline mutations (patients 274 and 209) also demonstrated the presence of a somatic mutation in the tumor. Three of the eight tumors

with somatic *bMLH1* alterations had mutations with uncertain pathological consequences; of these three, two were within introns (in patients 441 and 302) and one resulted in a silent change (in patient 298). These three mutations were analyzed with a splice-predictor program at the Berkeley Drosophila Genome Project Web site, and none of them were predicted to alter splicing. Of the remaining five mutations, four would be expected to result in truncated proteins (in patients 75, 53, 61, and 91), and one was a missense alteration occurring at a highly conserved codon (in patient 379).

All three tumors with an absence of hMSH2 expression had a germline *bMSH2* mutation (table 2, fig. 1), each of which would be expected to result in a truncated product. Patient 46 also showed the presence of a somatic alteration.

In addition to those alterations described above, six germline polymorphisms—four in *bMLH1* and two in *bMSH2*—were also noted (table 3). Three of the *bMLH1* polymorphisms and both of the *bMSH2* polymorphisms have been described elsewhere (for a list of published and unpublished mutations and polymorphisms, see the Web site of the International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer). The one unreported *bMLH1* sequence variant was a silent change in exon 17 (leucine to leucine) and was found in only a small number of cases.

The promoter regions of both *bMLH1* and *bMSH2*

Table 2**The Spectrum of Germline and Somatic Alterations in *hMLH1* and *hMSH2***

Gene, Mutation Type, and Patient Number	Exon/Intron	Nucleotide Change ^a	Consequence
<i>hMLH1</i> :			
Germline:			
274	I6	IVS5-1, g→a	Splice site
21	I9	IVS9+1, g→a	Splice site
83	E12	1321 G→A	A441T
209	E12	1217 G→A	S406N
Somatic:			
274	E7	583 del A	G201 stop
274	E19	2146 G→A	V716M
209	E8	677 G→A	R226Q
75	E6	469 del T	K160 stop
53	E7	583 del A	G201 stop
61	E17	1912 G→T	G638 stop
91	I16-E17	IVS17-7, del 17 bp	Splice site
379	E18	2000 A→G	D667G
298	E7	557 C→T	H186H
441	I8	IVS8+16, del a	Unknown
302	I11	IVS11+9, g→a	Unknown
<i>hMSH2</i> :			
Germline:			
46	I4	IVS4+1, g→a	Splice site
90	E8	1316-1317del CT	T441 stop
403	E13	2038 C→T	R680 stop
Somatic:			
46	E7	1165 C→T	R389 stop

^a Lowercase letters indicate nucleotides in intron; uppercase letters indicate nucleotides in exon.

were initially examined for hypermethylation through use of an *HpaII*-based PCR assay. Forty-four MSI-H tumors were examined, 42 of 48 for *hMLH1* and 2 of 3 for *hMSH2*. Hypermethylation of the *hMSH2* promoter was not detected for the MSI-H/*hMSH2*-negative tumors tested. Of the MSI-H/*hMLH1*-negative tumors, 34 demonstrated *hMLH1* hypermethylation by the *HpaII*-based PCR assay. Three MSI-H tumors lacked expression of *hMLH1* but had no detectable *hMLH1* mutations and did not exhibit *hMLH1* hypermethylation by the *HpaII* assay. We examined these 3 tumors, as well as another 17 that did show promoter hypermethylation by the *HpaII* assay, for methylation, using the MSP assay. The 3 tumors that did not show evidence of hypermethylation with the *HpaII* assay now showed evidence of methylation with the MSP assay, and the methylation status of the other 17 tumors was confirmed.

To further explore the molecular basis for the differences between the three discordant cases, the *hMLH1* 5' regions examined by the two assays (*HpaII* site, region 3, and MSP, region 2) were sequenced using bisulfite-modified DNA. For all three of these cases, all CpG sites were methylated, with the exception of the *HpaII* site. Thus, 37 of 42 tumors (88%) demonstrated promoter

hypermethylation for *hMLH1*. Four tumor samples had both mutations in *hMLH1* and hypermethylation (fig. 1). Two of these mutations were missense changes, and two were frameshifts; three were somatic, and one was a germline alteration. The one germline alteration was a missense change at a nonconserved site (in patient 83). All of the tested tumors lacking a germline or somatic alteration showed evidence of promoter hypermethylation (30/30). Thus, all of the MSI-H tumors tested that lacked *hMLH1* expression had either hypermethylation and/or a mutation in *hMLH1*.

Analysis of hMSH6

Three of four available tumors that lacked *hMSH6* expression were examined for the presence of a mutation in exon 5 of *hMSH6*. Patient 46 had a C insertion, and case 83 had both a C insertion and a C deletion within the C₈ repeat. Patient 19 showed the presence of an ins3405T mutation. All of these alterations are somatic, and all result in a frameshift.

Defective MMR and a Family History of CRC

Forty-seven (92%) of the MSI-H patients, all 18 (100%) of the MSI-L patients, and 160 (85%) of the MSS patients had family histories available for review. Of the 225 unrelated patients, 7 (3.1%) fulfilled the AC for HNPCC. Defective DNA MMR was noted in four of these seven individuals. Of the four patients with defective MMR, three were found to have germline defects—one in *hMLH1* (patient 274) and two in *hMSH2* (patients 46 and 403)—whereas the other tumor (in patient 19) demonstrated only *hMLH1* hypermethylation. It is possible that this tumor does indeed harbor a germline alteration not detected by our sequencing strategy. Notably, three of the seven tumors from patients with HNPCC did not exhibit evidence of defective DNA MMR. An additional two patients fulfilled the modified AC for HNPCC, only one of whom had an MSI-H tumor. No additional germline alterations were identified.

Table 3**Spectrum of Germline Sequence Variants in *hMLH1* and *hMSH2***

Gene and Exon/Intron	Nucleotide Change ^a	Consequence	Frequency
<i>hMLH1</i> :			
E8	655 A→G	I219V	19/48
I13	IVS13+14, g→a	Unknown	2/48
I14	IVS15-19, a→g	Unknown	27/48
E17 ^b	1959 G→T	L653L	4/48
<i>hMSH2</i> :			
I9	IVS10-9, t→a	Unknown	3/3
I12	IVS13-6, t→c	Unknown	1/3

^a Lowercase letters indicate nucleotides in intron; uppercase letters indicate nucleotides in exon.

^b Not previously reported.

The family characteristics of those patients with germline mutations are presented in table 4. Only three (patients 46, 274, and 403) of the seven patients with germline mutations fulfilled the AC. Two of these patients were aged <50 years (patients 35 and 44) at first diagnosis; the third was aged 55 years. Patient 403, who had a truncating *bMSH2* mutation, had a father who developed stomach cancer at age 38 years. Although patient 403 was not in a branch of the family that satisfied the AC, additional individuals on the paternal side of the family (aunt, uncle, cousin, and second cousin) had CRC, satisfying the AC for this family branch. We were not able to obtain family-history information for the one patient (patient 83) with a tumor that was associated with a germline missense *bMLH1* mutation. This was also the only tumor from a germline mutation-bearing patient that exhibited *bMLH1* hypermethylation. One additional patient (patient 21) had no documented family history, and two patients (patients 90 and 209) had no indication of a family history of either CRC or extracolonic HNPCC tumors.

Discussion

There has been considerable discussion about both the frequency of HNPCC and the means of identifying these individuals. The purpose of the present prospective study was to determine the frequency of inherited defects in DNA MMR (which we will refer to as "hereditary defective mismatch repair syndrome" or HDMMR), which is one particular cause of HNPCC. This was a prospective study in which all consenting surgical cases of CRC were examined for evidence of defective DNA MMR, without prior selection based on family history.

Overall, evidence of defective DNA MMR was found

in 20% of the 257 patients with CRC. This is within the range (12%–24%) previously published for colorectal cancer (Ionov et al. 1993; Thibodeau et al. 1993; Nakashima et al. 1997). All of the tumors with evidence of MSI-H (a phenotypic indicator of defective MMR) had altered protein expression of either hMLH1 or hMSH2. Abnormal protein expression for these two genes was not found in the remaining tumors. Furthermore, in the overall group of patients, absence of hMSH6 occurred only in the context of an abnormality in hMSH2 or hMLH1. Thus, it is likely that all cases of defective MMR that involve (a) hMSH2, (b) hMLH1, (c) hMSH6, and/or (d) an MSI phenotype have been identified in this group of patients.

Unequivocal pathogenic (truncating) germline mutations were identified in five patients, and missense germline mutations of unknown significance were identified in two additional patients. Thus, in this cohort of patients, the incidence of hereditary defective MMR is ~2%. This may be an underestimate, since we did not test for the presence of large deletions, which have been noted to occur (Wijnen et al. 1998). However, our findings suggest that the true frequency of HDMMR in this group of patients is not likely to be much higher, since somatic mutations and/or hypermethylation of the *bMLH1* promoter appears to account for the MSI-H phenotype in all of the remaining tumors. Although the Finnish study by Aaltonen et al. (1998) found a somewhat lower proportion of CRC to have defective DNA MMR (12% of cases), the frequency of germline *bMSH2* and *bMLH1* mutations was similar to that found in the present study. In the study by Aaltonen et al. (1998), tumors were screened for MSI but mutations were tested for in hMSH2 or hMLH1 only; hMSH6 was not examined. Additionally, immunohistochemical

Table 4

Family Characteristics of Patients with Germline Mutations

MUTATION TYPE AND PATIENT NUMBER	GERMLINE MUTATION	AGE AT ONSET (years)	MULTIPLE CRC IN PROBAND	NO. OF CRC CASES IN		NO. OF OTHER HNPCC CANCERS IN FAMILY ^a	TOTAL NO. OF CRC CASES IN FAMILY
				First-Degree Relatives	Second-Degree and Higher Relatives		
Pathogenic:							
274 ^b	<i>bMLH1</i> IVS5-1	35	Yes	4	3	0	8
21	<i>bMLH1</i> IVS9+1	68	No	2	NK	NK	3
46 ^b	<i>bMSH2</i> IVS4+1	44	No	1	7	0	9
90	<i>bMSH2</i> T441stop	35	No	0	0	0	1
403 ^b	<i>bMSH2</i> R680stop	55	No	0	4	0	5
Uncertain pathogenicity:							
83	<i>bMLH1</i> A441T	82	No	NK	NK	NK	NK
209	<i>bMLH1</i> S406N	65	No	0	0	0	1

NOTE.—NK = not known. In the case of patient 83, no family history was available, and, in the case of patient 21, only limited history was available.

^a Includes cancer of the endometrium, ovary, stomach, renal pelvis, ureter, and small bowel, in first-degree and higher relatives.

^b Patient fit the AC.

analysis was not included in their study. Cumulatively, these two studies suggest that the frequency of inherited defects in MMR in a population of patients with CRC referred for surgery is ~2%. The present study is the first large study in the United States that has looked at the frequency of disease in an unselected group of patients who were referred for CRC resection and in which no known founder mutations are described.

The germline mutations detected in the present study would be expected to produce a truncated or altered protein in all of the *bMSH2* and in two of the four *bMLH1* cases. The remaining two *bMLH1* alterations were missense changes; one of these mutations occurred in a highly conserved codon (S406N). Recent evidence indicates that some missense *bMLH1* mutations can affect the interaction of hMLH1 with hPMS2 (Guerrette et al. 1999); however, neither of the germline missense mutations identified in the present study occurred in this critical region. Nonetheless, the tumor with the S406N missense alteration also had a second somatic missense mutation, suggesting the possibility that this germline alteration may have functional consequences. The second missense alteration (A441T) occurred at a nonconserved codon. This tumor also exhibited hMLH1 promoter hypermethylation and, given the late age at onset for the patient (age 82 years), the A441T more likely represents a rare normal variant rather than a causative mutation. At this point, however, functional studies would be needed to distinguish between these two possibilities.

There is no clear consensus about how best to identify patients to be tested for HNPCC. Aaltonen et al. (1998) suggested that either family history, young age at diagnosis, or a history of multiple CRC or related cancers warranted testing for defective DNA MMR. This position was challenged in an editorial (Lynch and Smyrk 1998), which argued that family history alone may be the most appropriate means of identifying HNPCC. In the present study, tumors from three of the seven patients fulfilling the AC did not exhibit evidence of defective DNA MMR; germline defects were evident in only three of the patients with defective MMR. A fourth patient with defective MMR may have a mutation not detected by the methods employed in the present study. It is likely, therefore, that other genes not involving MMR are responsible for the colorectal cancer in the three patients with HNPCC who have normal MMR. Additionally, neither of the two additional patients satisfying the modified AC were found to harbor a germline alteration. Conversely, when the family characteristics of the patients with germline mutations were examined, only three of seven patients fulfilled the AC. It is becoming increasingly apparent that not all individuals fulfilling the AC or modified AC for HNPCC will have inherited defects in DNA MMR, and not all individuals

with inherited defects have a strong family history of cancer. HDMMR is a genetic diagnosis based on the finding of defective DNA MMR in the tumor and of a germline mutation in one of the DNA MMR genes, whereas HNPCC is a clinical diagnosis. Not all patients with HNPCC will have HDMMR, and not all patients with HDMMR will fulfill the AC (or other clinical criteria) for HNPCC. However, HDMMR is likely to be a more homogenous entity (despite allelic heterogeneity), unlike HNPCC, which is heterogeneous and probably includes cases of HDMMR, other hereditary forms of colorectal cancer (some for which the genes may not have identified), and the chance familial clustering of patients with sporadic colorectal cancer. It may be important to think about and treat these two syndromes as different entities. The Bethesda guidelines (Rodriguez-Bigas et al. 1997) may improve the predictive potential for identification of patients with defective DNA MMR genes over that provided by family history alone (Pistorius et al. 2000), since they take both the clinicopathological and family characteristics into account. We will report on the use of these guidelines, within this data set, in a future article.

Interestingly, all of the hMSH2-deficient cases were the result of a germline mutation. Although there are only three cases in the series, the data suggest that an absence of hMSH2 protein expression in CRC, when detected, is predictive of the presence of a germline mutation. Larger studies will be required to confirm such findings. It is also interesting to note that hMSH6 protein expression was lost in all three hMSH2 cases, but in only one of the hMLH1 cases. Our data suggest that, in such cases, the loss of hMSH6 is secondary to a mutation in either *bMLH1* or *bMSH2*, a result which supports the work of others (Wu et al. 1999; Planck et al. 2000). The complete concordance for loss of hMSH2 and hMSH6 is most likely due to the physical proximity of these two genes on chromosome 2. A germline mutation in *bMSH2* could be followed by loss of the second allele, by a gross chromosomal event. Such an event would also eliminate one normal *bMSH6* allele. Defective MMR resulting from loss of hMSH2 could then lead to instability of the hMSH6 poly C tract in exon 5 and subsequent inactivation of the second allele. As before, additional studies will be required to test this hypothesis.

In summary, whereas the frequency of defective DNA MMR in this referral-based study was found to be relatively common (20%), the frequency of inherited defects (HDMMR) was found to be low (~2%). The primary mechanism of DNA MMR gene inactivation appears to be epigenetic, with *bMLH1* hypermethylation occurring in 100% of hMLH1-negative MSI-H tumors without detectable mutations. Two conclusions can be drawn from the family history information. First,

although a strong family history correlates with the presence of germline mutations, it did not identify all the potential at-risk individuals. Second, with only four of seven patients with HNPCC having evidence of defective MMR (and none of those having a family history of HNPCC-related tumors), it is clear that there must be other factors that predispose to cancer development in these families. Molecular techniques can help in the identification of patients with defects in DNA MMR and can be used to complement family history and other guidelines in the clinical setting. This will greatly facilitate the diagnosis of HDMMR and the identification of individuals at high risk of cancer development.

Acknowledgments

We thank Dr. Gloria Petersen, for her helpful comments on the manuscript, and Karen Erwin, for secretarial support. This work was supported by National Institutes of Health grant R01 CA68535.

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

- Berkeley Drosophila Genome Project, <http://www.fruitfly.org/> (for splice-predictor program)
 International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer, <http://www.nfdht.nl/> (for listing of published and unpublished mutations and polymorphisms)
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for HNPCC [MIM 114500] and APC [MIM 175100])

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