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hMSH6 Alterations in Patients with Microsatellite Instability-Low Colorectal Cancer[1]

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ABSTRACT

Two microsatellite instability (MSI) phenotypes have been described in colorectal cancer (CRC): MSI-H (instability at >30% of the loci examined) and MSI-L (MSI at 1–30% of the loci examined). The MSI-H phenotype, observed in both hereditary nonpolyposis colon cancer-associated CRC and approximately 15% of sporadic CRC, generally results from mutational or epigenetic inactivation of the DNA mismatch repair (MMR) genes *hMSH2* or *hMLH1*. The genetic basis for the MSI-L phenotype, however, is unknown. Several other proteins, including hMSH3 and hMSH6, also participate in DNA MMR. Inactivating mutations of *MSH6* in yeast and human tumor cell lines are associated with an impaired ability to repair single-base mispairs and small insertion-deletion loops but not large insertion-deletion loops. This suggests that *hMSH6* mutations are more likely to be associated with a MSI-L phenotype than a MSI-H phenotype in CRC. To explore this possibility, we screened tumors from 41 patients with MSI-L CRC for *hMSH6* mutations with conformation-sensitive gel electrophoresis (CSGE) and for hMSH6 protein expression by immunohistochemistry. Alterations found with CSGE were confirmed by DNA sequencing of normal and tumor tissue. One somatic (Asp389Asn) and 15 germ-line changes were found. Of the 15 germ-line changes, 9 were found in an intron (none involving splice junctions), and 6 were found in an exon (Gly39Glu, Leu395Val, and 4 silent alterations). Immunohistochemical staining for hMSH6 performed on 34 of the 41 tumors revealed strong nuclear hMSH6 expression in all of the cases. Overall, our results suggest that *hMSH6* mutations do not play a major role in the development of sporadic CRC with a MSI-L phenotype.

INTRODUCTION

MSI[3] has recently been identified in tumors from patients with HNPCC and in a subset of various sporadic malignancies, including colorectal (CRC), endometrial, and stomach (1–5). In sporadic CRC, two distinct MSI phenotypes have been described: MSI-L (MSI at <30% of the loci examined) and MSI-H (MSI at ≥30% of the loci examined; 6–8). The MSI-H phenotype is associated with distinct clinicopathological features (for example, proximal tumor site, high grade, diploidy, favorable survival; 6, 9–11) and, in over 95% of the cases, is due to inactivation of *hMLH1* (90%) or *hMSH2* (5%; 6, 7, 12). The MSI-L phenotype, on the other hand, is not associated with distinct clinicopathological features or with altered hMLH1 or hMSH2 expression (6, 7). Currently, the biological basis of the MSI-L phenotype is not known. This phenotype could result from defects in MMR proteins other than hMSH2 and hMLH1 or from defects in other genes separate from those involved in MMR, or it could simply represent a "background" level of genetic instability that may be present in all tumors.

Several other proteins participate in DNA MMR, two of which are hMSH6 and hMSH3 (13–15). These two proteins independently form complexes with hMSH2 (16–18). The hMSH2-hMSH6 complex recognizes single-base mispairs and small (*i.e.*, single-base) insertion/deletion loops (19, 20), whereas the hMSH2-hMSH3 complex recognizes small and large insertion/deletion loops but does not appear to recognize single-base substitution mispairs (16, 17, 19, 21, 22). Yeasts that are deficient for MSH3 or MSH6 do not show marked MSI (21). However, yeasts that are deficient for both MSH3 and MSH6 exhibit the same level of MSI as observed in yeast that lacks MSH2 (21). Mice that are homozygous for a null mutation of *MSH6* develop gastrointestinal tumors and lymphomas (23). However, the tumors that develop in the mice do not exhibit significant MSI (23). Human tumor cell lines with *hMSH6* mutations exhibit MSI primarily in mononucleotide and not in dinucleotide repeats (20).

Germ-line mutations in *hMSH6* have been reported in a small number of HNPCC families (24–28). Some of the tumors that developed in these families were characterized by a MSI-H phenotype, whereas others were characterized by a MSI-L phenotype (25, 28). The presence of a MSI-H phenotype for some of these tumors would appear to contradict evidence in yeast and mice and in human cell lines that suggest that *MSH6* mutations should not be associated with such a phenotype. It should be noted, however, that the *hMSH3* gene was not entirely sequenced in any of the HNPCC families whose MSI-H tumors had germ-line *hMSH6* mutations. It is possible that a combination of *hMSH3* and *hMSH6* mutations were responsible for the MSI-H phenotype observed in these families. Risinger *et al.* (29) have demonstrated that an endometrial carcinoma cell line with MSI-H had a mutation in both the *hMSH3* and *hMSH6* genes. In one other study, however, Wu *et al.* (27) reported that 5 of 21 HNPCC (or suspected HNPCC) families with MSI-L tumors had germ-line *hMSH6* mutations.

The finding of germ-line *hMSH6* mutations in HNPCC families with MSI-L tumors and the observation that *MSH6* mutations in yeast and human tumor cells lines do not exhibit marked instability of large insertion-deletion loops suggest that *hMSH6* mutations may play a role in sporadic (*i.e.*, unselected) colorectal carcinoma with a MSI-L phenotype. In an effort to address the conflicting data reported thus far, we screened tumors from 41 patients with MSI-L CRC (unselected) for *hMSH6* mutations with CSGE and for hMSH6 protein expression by immunohistochemistry. Family history of colon cancer was not a selection criteria for these patients.

PATIENTS AND METHODS

Patients. The 41 patients in this study had CRC with a MSI-L phenotype (MSI at <30% of the loci examined; Refs. 6–8). These patients were obtained from 404 unselected CRC patients undergoing tumor resection at the Mayo Clinic. Frozen tumors and peripheral blood lymphocytes and/or normal colonic mucosa were the source of tumor and normal DNA, respectively, for these patients. Among the 41 patients, one patient had Crohn's disease, and one had chronic ulcerative colitis. There were 15 women and 26 men (ages, 36–83

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[3] The abbreviations used are: MSI, microsatellite instability; MSI-L, MSI-low; MSI-H, MSI-high; HNPCC, hereditary nonpolyposis colon cancer; CRC, colorectal cancer; MMR, mismatch repair; CSGE, conformation-sensitive gel electrophoresis; RT-PCR, reverse transcription-PCR.

Table 1 *Primer sequences*

Exon/Fragment	Sequence	Product size (bp)
1/1	5′-TTGGGCCTTGCGGCTGTC-3′/5′-CCCTCCGTTGAGGTTCTTCG-3′	347
1/2	5′-GCTGAGTGATGCCAACAAGGC-3′/5′-CAAGCGCCCCGCCGCTATG-3′	267
2	5′-TAATGCCAGAAGACTTGGAAT-3′/5′-AAAAAGTCTGCCTGTCTGTC-3′	377
3	5′-ACCCGGCCCTTATTGTTTAT-3′/5′-CTTCCCCCATCACCCTAA-3′	260
4/1	5′-TATTGTAGCTTGGGCGTAGC-3′/5′-ACCATTCTCTTCCGCTTTCG-3′	554
4/2	5′-GTGGGGGATAGTGAGAGTG-3′/5′-ATTTCCCCACCTTGTAACAG-3′	451
4/3	5′-CCTGATCACCCCGATTTTGA-3′/5′-TCCCTCCTCACCACTCTAT-3′	380
4/4	5′-GCATTTGGCCGTTATTCAGA-3′/5′-TGTACTGGGGGATAGTGTG-3′	386
4/5	5′-TCAGATGATCGCCATTGTTC-3′/5′-AGAGGCCCAATTCACTTTTC-3′	325
4/6	5′-TGGCATTGGGGTGATGTTAC-3′/5′-TGGCATTGGGGTGATGTTAC-3′	373
4/7	5′-AGAGAGGGTTGATACTTGCC-3′/5′-ACTTCTTCCATGATCCCTATAA-3′	357
4/8	5′-GCAGGGCTATAATGTATGAAG-3′/5′-TCTGTTGCGCTGTTTCTCTA-3′	365
4/9	5′-AGCAGGCTTTGACTCTGATT-3′/5′-CCAACACTGCGATACACTCT-3′	383
4/10	5′-ATTTGCCAGAAGAATACGAGT-3′/5′-CTGGCAAACAGCACTACTTAT-3′	262
5	5′-TGAAGCCTCACTTTTACCCT-3′/5′-GTCACTGGCTGACTTTTATGT-3′	406
6	5′-TAAGGGTTCATAAGAAAGACAA-3′/5′-TTAAAGGCTCATATACAAGAAG-3′	283
7	5′-GCAAAATGAGTATTCATTTGTG-3′/5′-CTTCAAATGAGAAGTTTAATGTC-3′	399
8	5′-TTGTGGCACAGACCGATAG-3′/5′-CAGAAGTGCCCTCTCAAAAA-3′	577
9	5′-TGAGAGGGCACTTCTGTTG-3′/5′-TCCCTTCCCCTTTTACTGTT-3′	328
10	5′-AAGGGATGATGCACTATGAAAA-3′/5′-TAAAACAGCCTGAAAGTGTATG-3′	369
For RT-PCR	5′-AGAATGCTTTTAGACGTGGAT-3′/5′-GCTATTGCCGTCCCATCAAAT-3′	
Northern-blot probe	5′-GCATTTGGCCGTTATTCAGA-3′/5′-ACTTCTTCCATGATCCCTATAA-3′	

years; median age, 70; mean age, 66.8). DNA from 50 normal blood bank donors were used to determine the allelic frequency for several of the alterations found in the *hMSH6* gene.

Microsatellite Analysis. DNA extraction and microsatellite analysis were performed as described previously (6, 12, 30). Tumors had previously been characterized for MSI with the following markers. For 31 of the 41 patients, up to 36 microsatellite markers were used to test for MSI. These markers included: *ACTC*, *TP53*, D5S107, D5S346, D6S251, D6S257, D6S258, D6S262, D6S264, D6S271, D6S289, D6S305, D7S480, D7S486, D7S490, D7S496, D7S510, D7S513, D7S518, D7S633, D7S655, D7S677, D17S261, D18S34, D19S177, D19S244, D19S245, D7S523, *MCC*, *TNF-α* (dinucleotide markers); DCC, D22S11 (trinucleotide markers); and CFTR2, D6S477, D6S503, D19S253 (tetranucleotide markers). For the remaining 10 patients, seven microsatellite markers were used to test for MSI. These markers included D18S49, D18S61, D18S34, *ACTC*, *TP53*, and D5S346 (dinucleotide markers) and BAT26 (mononucleotide marker). An average of 25.4 and 6.7 markers/tumor provided successful PCR results (range, 9–36 and 6–7) in each subgroup, respectively. The average percentage of markers demonstrating MSI in the 31 patients, in which up to 36 microsatellite markers were used, was 7.0% (range, 2.8–14.7%). In the 10 patients in which seven microsatellite markers were used to test for MSI, the average percentage of markers demonstrating MSI was 19.3% (range, 14.3–28.6%). A total of 67 markers exhibited MSI in the 41 patients.

Personal and Family History. Personal and family histories of cancer were obtained from pedigrees determined by genetic counselors in the Department of Medical Genetics ($n = 10$) or by chart review when a pedigree was not available ($n = 31$). Tumors were staged according to the Astler-Coller classification (31).

CSGE. Twenty primer pairs were used to screen the coding region and exon/intron junctions of the *hMSH6* gene (GenBank numbers U73732-U73737) for mutations using CSGE (32). The sequences of the primers used for CSGE (29) and DNA sequencing are shown in Table 1. PCR reactions were performed in 20-μl reaction mixtures containing 2.5–12.5 ng of template DNA, 16 pmol of each oligonucleotide primer pair, 1 unit of AmpliTaq Gold DNA polymerase (Roche Molecular System, Inc., Branchburg, NJ), 2 mM $MgCl_2$, 4 μl of 5× PCR buffer (containing 1.0 mM dATP, dGTP, and dTTP and 0.2 mM dCTP; Roche Molecular System, Inc., Branchburg, NJ) and 0.1 μl of [α-^{33}P]dCTP (3000 Ci/mmol, 10 mCi/ml; Amersham Pharmacia Biotech, Piscataway, NJ). The thermal cycling conditions were: heat activation step of 10 min at 94°C; 35 cycles of 94°C (30 s), 58°C (30 s), and 72°C (30 s); and a final 10-min extension at 72°C. Before electrophoresis, an 8-μl volume of loading buffer [30% glycerol (vol/vol), 0.25% bromphenol blue (wt/vol), and 0.25% Xylene cyanole (wt/vol)] was added to the PCR products. Using a thermal cycler, the mix was then heated to 95°C for 5 min, cooled to 68°C (decrease of 0.66°C/min), incubated for 30 min at 68°C, and cooled to 40°C (decrease of 0.66°C/min). Using a standard DNA-sequencing gel apparatus, we prepared a 1-mm thick gel with 10% polyacrylamide and a 99:1 ratio of acrylamide to bis-acrylamide (Bio-Rad Laboratories, Hercules, CA), 10% (vol/vol) ethylene glycol, and 15% (wt/vol) formamide in 0.5× TTE buffer [88.8 mM Tris, 28.5 mM Taurine, and 0.2 mM EDTA (pH 9.0)]. The running buffer was 0.5× TTE. The gel was pre-electrophoresed at 750 V for 1 h, and the samples were electrophoresed at 400 V for 14 h at room temperature.

DNA Sequencing. DNA sequencing was performed on samples with abnormal CSGE patterns using the Amersham Thermo Sequenase DNA sequencing kit (Amersham Life Science, Cleveland, OH) according to the manufacturer's specifications. DNA fragments were sequenced in both directions using the same primers that were used to perform PCR amplification (Table 1).

RNA Extraction, Reverse Transcription, and Northern Blot Analysis. RNA was extracted according to the Qiagen protocol (RNeasy, Qiagen, Santa Clarita, CA). Reverse transcription was performed in a 20-μl volume containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.125 mM each dNTP, 0.5 μg of oligo(dT), 2.5 μM hexamer, 200 units of Superscript II reverse transcriptase (Life Technologies, Inc., Gaithersburg,

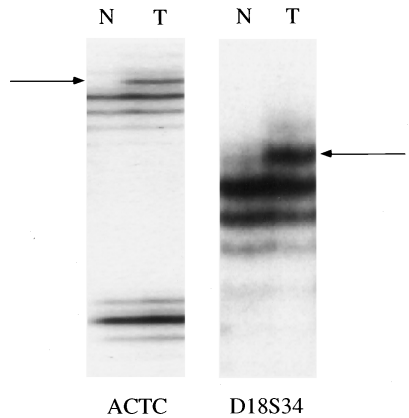

Fig. 1. Representative examples of MSI in colorectal tumor DNA from patients in this study. *On the left*, an example of MSI at the ACTC marker for patient 7; *on the right*, an example of MSI at the D18S34 marker for patient 18. *Arrows*, new alleles present in tumor DNA but not normal DNA.

Table 2 *Germline alterations of hMSH6*

Alteration no.	Location	Base change[a,b]	Consequence	Allele frequency[c]
1[d]	Exon 1	116 (G→A)	Gly39Glu	0.01
2	Exon 1	186 (A→C)	None	0.14
3	Exon 2	276 (G→A)	None	0.01
4	Exon 3	540 (C→T)	None	0.17
5	Exon 4	642 (T→C)	None	0.06
6	Exon 4	1186 (C→G)	Leu395Val	0.01 (0.02)
7	Intron 1	−36 (A→G)	None	0.09
8	Intron 2	+14 (C→G)	None	0.01
9	Intron 3	−54 (C→T)	None	0.08
10	Intron 5	+14 (A→T)	None	0.21
11	Intron 5	−16 (C→T)	None	0.01
12	Intron 7	−64 (gtttt deletion)	None	0.01
13	Intron 7	−34 (tttgttttaattccttt deletion)	None	0.08 (0.14)
14	Intron 9	−9 (tt deletion)	None	0.01
15	Intron 9	−9 (ttt insertion)	None	0.01

[a] The nucleotide designations are as provided in GenBank (Accession numbers U73732–U73737).
[b] For intronic alterations, a (+) sign is used to indicate the distance of the alteration from the 5′ end of the intron and a (−) sign is used to indicate the distance of the alteration from the 3′ end of the intron.
[c] Frequency of alteration in the 41 MSI-L patients. The frequency of this alteration in 50 normal blood bank donors is indicated in parentheses for two of the alterations.
[d] Previously reported as a common polymorphism (35).

MD), 20 units of RNase inhibitor (Boehringer Mannheim, Indianapolis, IN), and 1 μg of total RNA. Reverse transcription was performed at 38°C for 90 min followed by a reverse transcriptase denaturation step at 90°C for 5 min. Primers used to amplify exon 8 of the *hMSH6* cDNA are shown in Table 1. The upstream and downstream PCR primers for exon 8 amplification were located in exon 7 and 9 (Table 1). The PCR conditions were the same as that described for DNA amplification. PCR products were run on an agarose gel. Northern blot analysis was performed as described previously (33). The probe used for Northern blot analysis was synthesized with primers to exon 4 (Table 1). The signal intensity of the *hMSH6* signal was normalized to that obtained with a β-actin probe.

hMSH6, hMSH2, and hMLH1 Immunohistochemistry. Immunohistochemical staining for hMLH1 and hMSH2 were performed as described previously (34). The antibody to hMSH2 (Clone FE11, 0.5 μg/ml, Oncogene Science, Cambridge, MA) is a mouse monoclonal antibody generated with a COOH-terminal fragment of the hMSH2 protein, whereas the hMLH1 antibody (clone G168–728, 1 μg/ml, PharMingen, San Diego, CA) is a mouse monoclonal antibody that was prepared with full-length hMLH1 protein. The procedure for hMSH6 immunohistochemistry was the same as that used for hMLH1 and hMSH2 staining (34). The antibody to hMSH6 (Clone 44, 0.5 μg/ml, Transduction Laboratories, Lexington, KY) is a mouse monoclonal antibody generated with an NH_2-terminal fragment (codons 225–333) of the hMSH6 protein. Normal colonic epithelium and lymphocytes exhibit strong nuclear staining for hMSH2, hMLH1, and hMSH6 and, thus, served as positive internal controls for the staining of these proteins.

RESULTS

We screened the *hMSH6* gene for germ-line and somatic alterations in 41 patients having a MSI-L CRC (representative examples of MSI

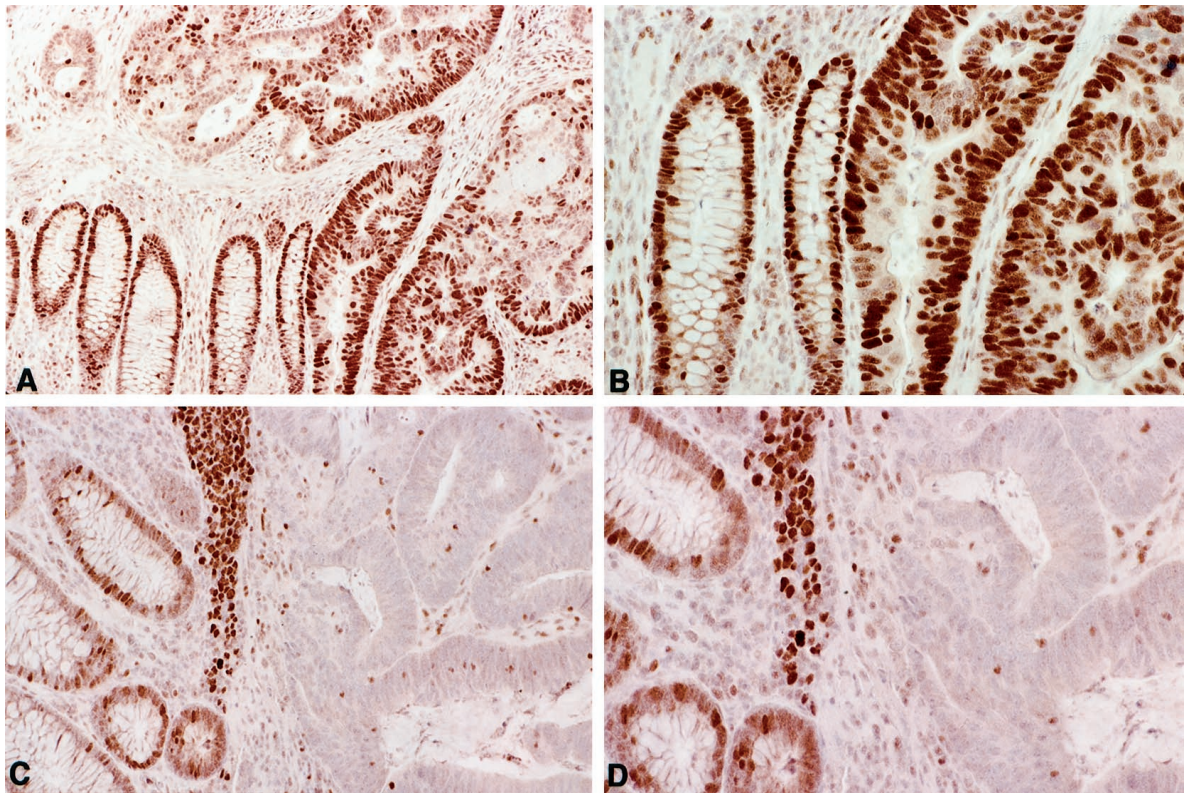

Fig. 2. Representative examples of positive (*A* and *B*) and negative (*C* and *D*) immunohistochemical staining for hMSH6 (*A* and *C*, × 200; *B* and *D*, × 400). All of the MSI-L tumors in this study exhibited positive (nuclear) staining for hMSH6. The tumor shown in *C* and *D* was a MSI-H tumor that also exhibited loss of hMSH2 expression.

Table 3 *Clinicopathologic findings, hMSH6 immunohistochemistry, and hMSH6 alterations in the 41 patients with MSI-L CRC*[a]

Pt. no.	MSI[b]	hMSH6 alteration[c]	hMSH6 IHC[d]	Gender (age[e])	Tumor site[f]	Stage	Personal history of cancer (other than CRC)	Family history of cancer[g]
39	2/18	4, 13	+	M (74)	Rec	C2		Mother, colon (43) Uncle[m], colon
12	1/7		+	M (68)	Cec	B2		Father, colon (64) Brother, gastric (45) Brother, prostate (75) Sister, unknown cancer
3	2/7		+	M (39)	Sig	C2		Grandfather[m], colon (65) Grandfather[p], colon (68) Father, prostate Sister, skin (19)
9	1/6		+	F (56)	Desc	C2		Father, colon (65) Uncle[p], colon (68)
11	2/7	10, 13, 14	+	M (50)	Asc	C2		Brother, colon (52) Grandfather, colon (78)
33	1/28	2	+	F (64)	Cec	C2		Father, colon (78) Sibling, colon
8	1/7		+	M (47)	Cec	B2		Uncle[m], gastric Uncle[m], gastric (90) Grandmother[m], gastric Aunt, bladder (47) Aunt, breast (78) Aunt, breast (72) Uncle, prostate (91)
10	2/7	4, 12	+	M (53)	Rec	D		Father, gastric (82) Uncle[p], gastric (95) Uncle[p], gastric (86) Mother, ovarian (78) Uncle, lung (59) Uncle, prostate (82) Uncle, kidney (45) Uncle, brain
18	1/33	8, 10	+	F (77)	Cec	B2	Breast cancer and benign uterine tumor	Brother, colon Father, gastric
5	1/7	8, 10	ND	M (76)	Rec	B2		Father, colon (70)
25	5/33	**1**, 2, 4, 5, 9, 13	ND	M (80)	Asc	B2		Sister, colon
32	2/29		+	F (70)	Cec	D		Brother, colon Mother, esophagus
44	1/16	2	+	M (52)	Rec	C1		Father, rectal (40)
40	1/18	2, 10	+	F (77)	Rec	B2		Father, gastric (75) Mother, unknown type cancer (67) Sibling, Hodgkin's lymphoma
7	2/7	7, 10	ND	M (63)	Sig	B1	Bladder	Mother, pancreas (42) Aunt[m], pancreas (72) Aunt[m], pancreas (58) Cousin, prostate
14	1/6	2	+	M (70)	Cec	B2		Father, skin Uncle, leukemia (82)
16	2/33	9, 10	+	F (68)	Rec	C2		Mother, unknown type of cancer
21	4/33	4, 5, 9	+	M (81)	Sig	B1	Prostate cancer	Mother, unknown type of cancer Father, unknown type of cancer Sister, unknown type of cancer Sister, unknown type of cancer
26	3/33	10, 11	+	M (76)	Cec	B1		Father, lung cancer
27	1/29		+	M (75)	Rec	B1		Brother, lung Sister, liver
28	2/29	4, 10	+	M (71)	Rec and Sig	C2	Synchronous colon cancers	Father, bladder
29	1/29	4, **6**, 10	+	M (76)	Sig	D	Squamous cell carcinoma	Father, squamous cell carcinoma
31	2/29	3, 4, 6, 7, 9, 10, 13	+	F (81)	Asc	C2	Breast	Brother, brain
34	1/28	2, 8	+	F (79)	Asc	D	Breast	Mother, breast Brother, bone
42	1/17	7, 10, 15	+	M (73)	Rec	B2		One relative, leukemia
15	3/33	9	ND	M (44)	Rec	C2		No family history of cancer
2	1/7	2	ND	M (83)	Cec	C2		No family history of cancer
45	1/16	7, 10	+	M (41)	Cec	B2	Myeloproliferative disease	No family history of cancer
17	1/33	7, 10	+	F (57)	Sig	C2		No family history of cancer

Table 3 *Continued*

Pt. no.	MSI[b]	hMSH6 alteration[c]	hMSH6 IHC[d]	Gender (age[e])	Tumor site[f]	Stage	Personal history of cancer (other than CRC)	Family history of cancer[g]
22	3/33	4, 13	+	M (75)	Sig	B1		No family history of cancer
23	3/33	4, 7	+	M (80)	Rec	D		No family history of cancer
24	1/33	4, 5, 9, 13	+	F (60)	Trans	B2		No family history of cancer
36	1/19	2, 10	+	M (78)	Asc	C1		No family history of cancer
38	1/18		+	F (69)	Cec	D		No family history of cancer
43	1/17	2, 10	ND	F (69)	Sig	B1	Melanoma	No family history of cancer
46	1/11	2, 7, 10	+	F (80)	Unk	D		No family history of cancer
19	1/33	2	+	F (54)	Sig	B1		Adopted
20	1/33	10	+	F (36)	Rec	B1		No information
30	1/29	4, 5, 9, 13	ND	M (65)	Rec	D		No information
35	1/19	2, 4, 10	+	M (81)	Sig	D		No information
41	1/18		+	F (70)	Rec	D		No information

[a] Patients with strongest family history of cancer are shown at the top of the table.
[b] MSI (number of markers exhibiting MSI/number of markers providing successful PCR results).
[c] See Table 2 for designations. Missense mutations indicated in bold.
[d] IHC, immunohistochemistry; ND, not done (no slides available for immunostaining).
[e] Age at colorectal cancer diagnosis.
[f] Cec, cecum; Asc, ascending colon; Trans, transverse colon; Desc, descending colon; Sig, sigmoid colon; Rec, rectum; Unk, unknown.
[g] Age at diagnosis shown in parentheses if known. For individuals with colorectal or gastric cancer, the side of the family is indicated as maternal (superscript "m") or paternal (superscript "p") if known.

are shown in Fig. 1) with CSGE (32). PCR fragments showing abnormal CSGE-banding patterns were then subjected to DNA sequence analysis. The CSGE and DNA sequencing results for a portion of exon 1 (bases 216–270) were not interpretable, presumably because of the high GC content (>70%) of this region.

One somatic and 15 germ-line DNA alterations were found in the 41 patients. The tumor from patient 45 had a somatic G to A missense mutation at nucleotide 1168, which led to a nonconservative replacement of Asp by Asn. Of the 15 germ-line DNA alterations observed (Table 2), 9 alterations occurred in an intron, and 6 occurred in an exon. None of the intronic alterations involved the consensus splice junctions. Of the six exonic changes, two were missense alterations (Gly39Glu and Leu395Val), and four were silent mutations (Table 2). The codon 39 alteration has been previously been reported as a common polymorphism (35). Screening of 50 normal blood bank donors identified two cases with the codon 395 alteration. Kolodner *et al.* (36) have recently shown that expression plasmids carrying the hMSH6 Leu395Val change complement the MMR defect of a msh3msh6 mutant strain, which suggests that this alteration is a polymorphism that does not affect MSH6 function.

One of the intronic alterations was a 17-bp deletion occurring in a 17-bp tandemly repeated sequence within the branch site of intron 7 (34 bp upstream from exon 8). Eight of the 41 MSI-L CRC patients and 14 of the 50 controls were heterozygous for this 17-bp deletion; no homozygotes were identified. Northern blot analysis revealed that the *hMSH6* mRNA, obtained from peripheral blood leukocytes, was normally expressed in patients with the 17-bp deletion (a sufficient amount of mRNA was not available from tumor tissue to perform the Northern analysis). Additionally, RT-PCR analysis with primers to exon 8 and mRNA obtained from tumor tissue revealed that the deletion did not affect RNA splicing (data not shown).

Paraffin-embedded tumor sections were available for hMSH6, hMSH2, and hMLH1 immunostaining for 34, 39, and 39 of the patients, respectively. All of the available tumor sections demonstrated normal nuclear expression for hMSH6 (Fig. 2, *A* and *B*), hMLH1, and hMSH2 (Table 3). An example of a tumor showing an absence of hMSH6 expression is shown in Fig. 2, *C* and *D*. This tumor exhibited high-level MSI (MSI-H) and was not one of the 41 MSI-L tumors identified for this study. The immunostain for this patient is shown simply as an example of a tumor that shows a loss of hMSH6 expression.

Details of the family history for each patient are shown in Table 3. Only patient 39 met the Amsterdam criteria (37) for HNPCC. An additional 13 patients had one or more close relatives with a history of colorectal or gastric cancer.

DISCUSSION

In this study, we have examined a group of unselected CRC patients with MSI-L tumors for germ-line and somatic *hMSH6* mutations. Although several studies have defined the criteria for MSI-L (6–8), we recognize the difficulty in identifying such cases. Depending on the number and type of markers being used, some tumors may differ in their MSI status, typically between MSS (microsatellite stable) and MSI-L. Additionally, PCR artifact may lead to false positive typing for MSI. Given the number of markers used for the majority of tumors in this study, our sampling is likely to be more inclusive rather than exclusive for the MSI-L phenotype. Even under these conditions, however, no unequivocally pathogenic germ-line *hMSH6* mutations were found, and all of the examined tumors showed normal levels of hMSH6 protein expression. However, we did observe 16 *hMSH6* alterations. Fifteen of these were germ-line changes and one was a somatic change. Only 2 of the 15 germ-line alterations led to a change in the primary sequence of the hMSH6 protein: Gly39Glu and Leu395Val, both of which are reported to be polymorphisms (35, 36). On the basis of immunohistochemical analysis, hMSH6 was normally expressed in the tumors from all of these patients (Table 3). The somatic Asp389Asn mutation and the germ-line Leu395Val alteration fall within a domain (amino acids 326–575 of hMSH6) that is important for contact of hMSH6 with hMSH2 (38). It is conceivable that these alterations could impair MMR by inhibiting the interaction of hMSH6 with hMSH2. However, protein-protein interaction studies such as those performed by Guerrette *et al.* (38) would have to be done to explore this.

The Gly39Glu change has previously been described by Nicolaides *et al.* (35) as a polymorphism that has a frequency of 11%. However, the size and composition of the normal population of patients used to determine the frequency of this alteration was not described. In our study, we find that this alteration is present in only 1 of the 41 MSI-L patients.

The 17-bp deletion observed in intron 7, 34 bases before exon 8, is located within the branch site of intron 7 and could conceivably alter RNA splicing. However, our data suggests that this alteration is likely

to be a polymorphism. Both Northern blot and RT-PCR analyses demonstrated normal splicing and normal expression in tissue from these patients. Moreover, immunohistochemical analysis revealed normal hMSH6 protein in tumors from these patients.

Although CSGE is a very sensitive method for mutation detection (32), we cannot exclude the possibility that other alterations might be present. We were able to screen the entire gene for mutations with the exception of codons 72–90 of exon 1. The high GC content (>70%) of this region made it difficult to adequately screen this portion of the gene with either CSGE or DNA sequence analysis. Additionally, we cannot exclude the possibility of larger deletions that cannot be identified by this technique. If other mutations are present, however, they likely represent a very low number since none of our cases demonstrated a lack of protein expression.

Our data differ from those of Wu *et al.* (27), who recently reported the presence of germ-line *hMSH6* mutations in 4 of 18 HNPCC families with MSI-L colorectal tumors. The mutations observed in that study were a Ser144Leu missense mutation (1 patient), a T insertion in codon 217, leading to a truncated hMSH6 protein (2 patients), and a Gln1258stop change (1 patient). The authors considered each of the latter two mutations pathogenic. The missense mutation was considered potentially pathogenic because it was a nonconservative change located in a conserved codon (39). Although we did not observe any of these alterations in our patient population, there is a fundamental difference between these two studies. We have examined an unselected series of MSI-L cases, whereas the cases in the report by Wu *et al.* are selected for HNPCC or a positive family history of colon cancer. Although germ-line mutations in hMSH6 may lead to MSI-L CRC, the majority of MSI-L sporadic CRC do not seem to have hMSH6 involvement (27).

Several reports have described the presence of germ-line *hMSH6* mutations in both typical and atypical HNPCC families (24–28). Wijnen *et al.* (28) found truncating germ-line MSH6 mutations in seven HNPCC families not fulfilling the Amsterdam criteria and in three typical HNPCC families. Of interest, these authors found that the atypical HNPCC families with germ-line MSH6 mutations had a high frequency of endometrial carcinoma compared with HNPCC families with germ-line MSH2 and MLH1 mutations. In the current study, only 1 of the 41 patients (patient 39) had a family history consistent with HNPCC, according to the original Amsterdam criteria (37). This patient had a 17-bp deletion in intron 7 and a silent alteration in exon 3. The 17-bp deletion does not seem to have functional significance, because it does not affect RNA expression, splicing, or hMSH6 protein expression and is found frequently in controls. An additional 13 patients had family histories suggestive of HNPCC, with either 1 or more close relatives having colon or gastric cancer (Table 3). Of these 14 patients, only 1 (patient 25) had a germ-line alteration of *hMSH6*. This patient had a Gly-to-Glu change at codon 39. As noted above, this alteration has previously been described as a polymorphism (35). The tumors from these patients have all been assessed for hMSH2 and hMLH1 expression, with all having normal expression. Previous studies have shown a high degree of concordance between loss of hMSH2 and hMLH1 expression and either mutational or epigenetic inactivation of *hMSH2* or *hMLH1* (12). Thus, it seems unlikely that any of these patients have germ-line mutations of *hMSH2* or *hMLH1*. This suggests either a chance clustering of HNPCC-associated tumors in these families or perhaps a role for other MMR or non-MMR genes in these families. Of note, based on the review of medical records, none of the family members in our series had endometrial cancer.

Overall, our results suggest that *hMSH6* inactivation does not play a major role in the etiology of unselected MSI-L CRC. Other MMR genes, such as *hMSH3*, or non-MMR genes will have to be examined for possible associations. Additionally, these results do not rule out the increased involvement of hMSH6 in selected cases of familial colorectal and/or endometrial cancer.

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