Tumor DNA microsatellite instability (MSI) is a consequence of defects in DNA mismatch repair. A high level of microsatellite instability (MSI-H) is recognized in a subset of patients diagnosed with Hereditary Nonpolyposis Colorectal Cancer (HNPPC) by pedigree, and in 10% to 15% of all colorectal cancers in most unselected series (1–3). Both somatic and germ line mutations have been identified in several putative genes from the mutS (MSH2, MSH3, and MSH6) and mutL (MLH1, MLH3, PMS1, and PMS2) gene families in colon cancer with defective mismatch repair (4–7).

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Germ line mutations in MSH2 and MLH1 account for the majority of familial cases of colon cancer with defective mismatch repair (8), whereas methylation of the MLH1 promoter has been implicated in the majority of sporadic tumors with the MSI-H phenotype (9, 10). Colorectal tumors are determined to be MSI-H by the presence of instability at ≥30% of evaluated microsatellite loci (11). Whereas the MSI-H phenotype identifies a defect in mismatch repair, it does not predict the involved gene and is unable to distinguish between somatic and germ line mutations and epigenetic causes. Immunohistochemical analysis is often able to identify a causative gene as mutations in MLH1, MSH2 or MSH6 or promoter hypermethylation of MLH1 typically results in loss of protein expression.

Some reports have suggested complete concordance between the MSI-H phenotype and loss of expression of MLH1 or MSH2 (12). However, normal immunohistochemical staining of MLH1 and MSH2 proteins in colorectal tumors with the MSI-H phenotype has since been recognized (13–16). This is an uncommon presentation, and the molecular pathogenesis for this discordance is poorly understood. Hypotheses include missense or subtle mutations of MSH2 or MLH1 which result in a translated nonfunctional protein that retains its antigenic epitope for immunohistochemical expression, failed antigen retrieval in archived tissue, or mutations of other DNA mismatch repair genes (17, 18).

Among pathogenetic germ line mutations identified in kindreds followed by the International Collaborative Group on HNPPC, MLH1 is responsible for ~45% to 50%, MSH2 for 35% to 40%, and MSH6 for 10%, with MSH3, PMS1, and PMS2 collectively accounting for fewer than 5% (19). The PMS2 and MLH1 proteins form the mutL heterodimer that functions as a component of the human mismatch repair complex. Until recently, only five families have been reported with PMS2 germ line mutations (20–24). Whereas two of these reports initially indicated heterozygous germ line mutations in PMS2, subsequent work suggests that in all five families there are mutations in PMS2 on both alleles, and the disease is recessive. The phenotype in such families is like other homozygous DNA mismatch repair cases with café au lait spots like seen in neurofibromatosis type 1, predisposition to hematologic malignancies, brain tumors (Turcot syndrome), and very early-onset colorectal cancers (25–28). Study of this gene has been hampered by the finding of a PMS2 pseudogene (24, 29, 30). However, a recent study, utilizing diploid to haploid conversion to facilitate the study of this gene, identified two individuals with heterozygous germ line PMS2 mutations among seven patients with selective loss of PMS2 by immunostaining of colorectal tumor (31). Thus, limited evidence currently supports the concepts of both monoallelic mutations and biallelic mutations in PMS2 predisposing to cancers, and these can be germ line or somatic mutations.

The overall contribution of loss of PMS2 expression (either by mutation or other mechanisms including methylation or chromosomal loss) to MSI1 colon cancers has been suggested but remains undefined (32). In this report, we describe the frequency of altered PMS2 protein expression within a rare subset of patients: those with MSI-H colorectal tumors and intact protein expression of MLH1, MSH2, and MSH6.

### Materials and Methods

**Tumor ascertainment.** Tumor specimens were identified from four cohorts: (a) The Mayo Clinic Cooperative Family Registry for Colon Cancer Studies (Colon CFR), which includes both randomly selected population-based patients diagnosed with colorectal cancer as well as clinic-based patients diagnosed with colorectal cancer under the age of 50 years and colorectal cancer patients with a family history of cancer; (b) The Australasia Colon Cancer Family Registry for Colon CFR, which includes randomly selected population-based patients with colorectal cancer diagnosed before the age of 60 years as well as the probands of colorectal cancer family clinic-based patients. The Colon CFR is a National Cancer Institute–supported consortium established in 1997 to create a multinational comprehensive collaborative infrastructure for interdisciplinary studies in the genetic epidemiology of colorectal cancer (detailed information about the Colon CFR can be found at [http://epi.grants.cancer.gov/CFR/](http://epi.grants.cancer.gov/CFR/)). (c) Consecutive cases referred to the Mayo Clinic Molecular Genetics Laboratory for HNPCC screening from September 1998 to September 2002; and (d) patients enrolled from April 1993 to January 1998 to the North Central Cancer Treatment Group (NCCTG) randomized trial of adjuvant chemotherapy for B2 and C colon cancer (33). Because patients in the first two groups noted above were part of a National Cancer Institute–supported registry, more detailed personal and family history information is available.

**Fig. 1.** A, MSI-H colon adenocarcinoma with loss of PMS2 expression. Nuclear staining of benign colonocytes and stromal cells (e.g., lymphocytes) serves as internal positive control. B, adenocarcinoma with normal PMS2 expression.
A three- to four-generation pedigree was constructed for all individuals enrolled in the Australasia and Mayo CFR by a study coordinator and reviewed by a Medical Geneticist with the intent of eliciting all information related to cancer in the family. For those patients in group 3 (clinical referrals), more limited family history information was provided by the referring physician. Additional information, when required, was obtained by a genetic counselor. Information for the last group of patients (NCCTG) was limited to gender, age, and tumor characteristics. For both of these later two groups, testing was confined to tumor analysis for MSI and immunohistochemistry. Testing for germ line mutations was not possible.

**Microsatellite instability.** All included cases were tested for MSI. DNA was extracted from paraffin-embedded tissues as previously described (2). For two of the patient cohorts (Colon CFR and clinical), paired normal and tumor DNA were amplified by PCR using a panel of 10 microsatellite markers—five dinucleotide markers (D5S346, D17S250, ACTC, D18S55, and D10S197), four mononucleotide markers (BAT26, BAT25, BAT40, and BAT34C), and one penta-mono-tetra compound marker (MYCL). For the NCCTG group of patients, one mononucleotide marker (BAT26) and 16 dinucleotide markers (ank1, D8S1055, D8S133, D8S136, D8S1720, D8S1742, D8S1760, D8S1820, D8S1842, D8S1925, D8S254, D8S261, D8S262, D8S560, and MYCL) were used. As described by Thibodeau et al. (34), MSI was defined as the presence of novel fragments in the PCR-amplified product of tumor DNA compared with normal DNA. Tumors were designated as MSI-H if ≥30% markers showed instability (11).

**Immunohistochemistry.** Immunohistochemical analysis for MLH1, MSH2, and MSH6 expression had been done on all MSI-H tumors using methods described elsewhere (2, 15). In brief, formalin-fixed, paraffin-embedded tissues were sectioned at 6 μm and stained with antibody to MLH1 (BD Pharmingen, San Diego, California), MSH2 (Oncogene Science), and MSH6 (Transduction Laboratories, BD Pharmingen). Neoplastic cells with absent nuclear staining were interpreted to have an absence of protein expression. Intact nuclear staining in surrounding nonneoplastic cells was required to serve as an internal positive control.

MSI-H tumors identified to have intact expression of MLH1, MSH2, and MSH6 were then studied for PMS2 protein expression using a purified mouse anti-PMS2 monoclonal antibody (clone A16-4, BD PharMingen; Fig. 1). All Mayo cases were assessed by a single pathologist (J.L.B.), whereas Australasia cases were assessed by two pathologists (J.L.B. or A.J.R.R.). Note that PMS2 expression is nearly always lost when MLH1 is not expressed. This study focused only on cases with isolated PMS2 loss of expression.

### Results

A total of 2,719 colorectal tumors were studied, including 1,497 samples from Colon CFRs (Mayo and Australasia), 690 samples from the clinical testing cohort, and 532 samples from the NCCTG adjuvant cohort. There were 535 tumors (19.7%) that satisfied MSI-H criteria (Table 1). Among the MSI-H tumors, the vast majority (499 of 535, 93%) had loss of expression for at least one of the proteins for MLH1, MSH2, and MSH6, whereas intact immunostaining for all three protein products was identified in only 38 MSI-H tumors (7.2%). Available clinical data for patients in this subgroup are summarized in Table 2. Immunohistochemistry for PMS2 was technically unsuccessful in 6 of these 38 cases. Of the remaining 32 cases, loss of PMS2 expression was observed in 23 tumors (72%). Of these cases, clinical data were available for 22 patients, and detailed family histories were available for 19 patients: 12 from the Australasia CFR, 4 from the Mayo CFR, and 3 from the clinical referral cases (see Materials and Methods). One family met Amsterdam I Criteria for HNPCC syndrome and one met Amsterdam II Criteria (Table 3; ref. 35).

### Discussion

In our series, loss of PMS2 expression (and possibly function) is present in 72% of cases of colorectal tumors (23 of 32) with an MSI-H phenotype but in which there is retention of normal expression of MLH1, MSH2, and MSH6 DNA mismatch repair gene products. Selective loss of PMS2 (i.e.,

### Table 1. Results

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Colon CFR</th>
<th>Mayo Clinic</th>
<th>NCCTG</th>
<th>Colon CFR</th>
<th>Mayo Clinic</th>
<th>NCCTG</th>
<th>Colon CFR</th>
<th>Mayo Clinic</th>
<th>NCCTG</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of tumors</td>
<td>689</td>
<td>690</td>
<td>532</td>
<td>492</td>
<td>316</td>
<td>535</td>
<td>38 (7.2%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSI-H</td>
<td>138 (20.2%)</td>
<td>114 (16.5%)</td>
<td>59 (11.1%)</td>
<td>2 (3.4%)</td>
<td>8 (16%)</td>
<td>175 (55%)</td>
<td>38 (7.2%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSI-H with intact MLH1, MSH2, and MSH6</td>
<td>7 (5.1%)</td>
<td>10 (8.8%)</td>
<td>2 (3.4%)</td>
<td>8 (16%)</td>
<td>11 (6.3%)</td>
<td>38 (7.2%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Characteristics of patients with MSI-H colorectal cancer and intact MLH1, MSH2, and MSH6 by immunohistochemistry

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total with intact MLH1, MSH2, and MSH6 (n = 35)</th>
<th>PMS2 negative (n = 22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, y (range)</td>
<td>47.7 (13-67)</td>
<td>47.5 (28-67)</td>
</tr>
<tr>
<td>Male</td>
<td>60%</td>
<td>64%</td>
</tr>
<tr>
<td>Proximal colon cancer*</td>
<td>59%</td>
<td>52%</td>
</tr>
<tr>
<td>Poor differentiation</td>
<td>26.5%</td>
<td>50%</td>
</tr>
</tbody>
</table>

*Clinical data available on 28 of 35 cases with intact staining for MLH1, MSH2, and MSH6.
† Data available on 33 of 35 cases. Note that 1 of 19 from Australasia was poorly differentiated whereas 8 of 15 from United States were poorly differentiated.
there was normal expression of the other three proteins tested) was a rare phenotype, occurring in only 4.3% (23 of 529) of all MSI-H tumors evaluated from a total of 2,719 colorectal cancers tested. Note that PMS2 loss of expression is consistently seen whenever MLH1 expression is lost. This relationship, related to the mutL heterodimer formed by these two DNA mismatch repair proteins, is not the subject of this study. The frequency of selective loss of PMS2 differed among the patient population tested, with the lowest frequency detected in the NCCTG group. Selection criteria for the NCCTG group were not based on family history or age of onset and did not exclude older cases (as was done for the Australasia population-based series), and thus, this group was most likely to have the lowest frequency of inherited cases. This low frequency of PMS2 involvement in this group is consistent with other studies examining the frequency of mismatch repair protein loss in colon cancer. In general, the loss of MLH1 and MSH2 accounts for nearly 100% of the MSI-H cases in sporadic colon cancer (10). The highest frequency of selective loss of PMS2 in the American cohorts occurred in the clinical group, where the selection criteria were almost exclusively because of family history or young age of onset. The Australasia population-based group (all under age 60) had the overall highest percent of selective PMS2 loss. Although not statistically significant, these data indirectly suggest that the loss of PMS2 in this study is more likely to be linked to a germ line mutation rather than to the presence of a somatic etiology, as the rate of selective loss of PMS2 was lowest in the NCCTG group, the only group not selected for known risk factors for hereditary disease (young age and/or family history).

Loss of PMS2 expression could reflect germ line alterations or somatic inactivation of PMS2. Alternatively, mutations in

<table>
<thead>
<tr>
<th>ID number</th>
<th>Primary, age of onset, gender</th>
<th>Affected relative: primary (age of onset)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Ascending colon, 37, F</td>
<td>Maternal cousin: sigmoid (45)</td>
</tr>
<tr>
<td>4</td>
<td>Cecum, 60, M</td>
<td>Sister: breast (58); Brother: leukemia (55); Niece: ovary (22)</td>
</tr>
<tr>
<td>5</td>
<td>Splenic flexure, 43, M</td>
<td>Father: prostate (57); Mother: uterus (56), breast (59); Paternal grandfather: prostate (71); Paternal grandmother: breast (87)</td>
</tr>
<tr>
<td>6</td>
<td>Ascending colon, 42, F</td>
<td>Maternal grandfather: lung (58); Maternal aunt: leukemia (38); Maternal cousin: breast (39)</td>
</tr>
<tr>
<td>8</td>
<td>Rectum, 51, M</td>
<td>Bladder, 47</td>
</tr>
<tr>
<td>9</td>
<td>Left colon, 48, F</td>
<td>Mother: cervical ca (N/A)</td>
</tr>
<tr>
<td>10</td>
<td>Ascending colon, 55, M</td>
<td>N/A</td>
</tr>
<tr>
<td>12</td>
<td>Sigmoid colon, 39, F</td>
<td>N/A</td>
</tr>
<tr>
<td>13</td>
<td>Transverse colon, 28, M</td>
<td>Maternal grandfather: colon (76); Paternal great-uncle: colon (N/A); Paternal grandmother: ovary (76)</td>
</tr>
<tr>
<td>16</td>
<td>Ascending colon, 53, M</td>
<td>Daughter: von Hippel-Lindau; Paternal aunt: breast (early 40s); Paternal uncle: prostate (81)</td>
</tr>
<tr>
<td>A1</td>
<td>Transverse colon, 49, M</td>
<td>Mother: colon (76), uterus (60); Maternal grandfather: intestinal tract (66); Paternal grandmother: colon (44); Paternal uncle: esophagus (61); Paternal uncle: prostate (70)</td>
</tr>
<tr>
<td>A2</td>
<td>Descending colon, 40, F</td>
<td>Mother: intestinal tract (39 (46); Paternal grandfather: unknown primary (80)</td>
</tr>
<tr>
<td>A4</td>
<td>Colon, 67, M</td>
<td>Brother: colon (69); Sister: cecum (72); Sister: colon (68)</td>
</tr>
<tr>
<td>A5</td>
<td>Intestinal tract, 36, F</td>
<td>Mother: intestinal tract (39), stomach (39), ovary (39); Sister: breast (44), liver (44)</td>
</tr>
<tr>
<td>A6</td>
<td>Transverse colon, 59, M</td>
<td>Niece: kidney (53), skin (40), liver (54); Mother: stomach (N/A); Father: bone marrow (N/A); Niece: cervix (N/A); Niece: breast (30); Sister: stomach (67); Brother: intestinal tract (48)</td>
</tr>
<tr>
<td>A8</td>
<td>Sigmoid colon, 44, M</td>
<td>Mother: gallbladder (52); Father: rectum (52); Maternal grandfather: rectum (52); Paternal grandmother: lung (92), bone marrow (93); Sister: uterus (37); Maternal aunt: bone (56), thyroid (54), intestinal tract (49), uterus (50); Maternal cousin: breast (50); Maternal cousin: rectum (40), ascending colon (N/A); Paternal aunt: rectosigmoid junction (52)</td>
</tr>
<tr>
<td>A9</td>
<td>Ascending colon, 39, M</td>
<td>Paternal grandfather: lung (64)</td>
</tr>
<tr>
<td>A10</td>
<td>Rectum, 43, M</td>
<td>N/A</td>
</tr>
<tr>
<td>A11</td>
<td>Transverse colon, 47, M</td>
<td>Paternal grandfather: ampulla of Vater (56), stomach (56); Paternal uncle: kidney (66), brain (76); Paternal uncle: lung (63)</td>
</tr>
<tr>
<td>A12</td>
<td>Sigmoid colon, 60, F</td>
<td>Father: intestinal tract (33); Maternal uncle: brain (60); Maternal uncle: stomach (53)</td>
</tr>
<tr>
<td>A13</td>
<td>Rectum, 45, M</td>
<td>Mother: intestinal tract (70); Father duodenum (59); Maternal grandmother: skin (65); Maternal grandfather: prostate (80); Paternal grandmother: breast (80); Paternal uncle: prostate (61); Paternal uncle: prostate (64)</td>
</tr>
<tr>
<td>A14</td>
<td>Cecum, 48, F</td>
<td>Father: prostate (73); Paternal grandfather: prostate (82)</td>
</tr>
</tbody>
</table>

Abbreviation: N/A, information not available.
MLH1 can lead secondarily to loss of PMS2 expression while retaining MLH1 immunohistochemical expression (36). Thus, at a clinical level, addition of PMS2 immunohistochemistry may also help identify families with hereditary MLH1 mutations. Germ line testing for PMS2 and MLH1 mutations was not conducted in this study as germ line DNA or required informed consent was not available for many of the cases (clinical referrals and NCCTG group of patients), and sequencing of PMS2 has been difficult to perform.

The clinical features of cases with loss of PMS2 are shown in Table 2. As with deficiencies of the other DNA mismatch repair genes, right-sided tumors were more common than in the overall colorectal cancer population. This possibly could be due to an artifact of study design, as physicians may have been more likely to order clinical MSI testing if a tumor was right sided. Similarly, the age at diagnosis is two decades younger than overall colon cancer patients, but the Colon CFR emphasized recruitment of young individuals with colorectal cancer, and clinical testing again would select for young cases such as those that met the Bethesda guidelines (37). An extremely large study or the pooling of the world’s known carriers will be required to fully address the clinical correlates of selective loss of PMS2 expression in MSI-H tumors in the general population, as these phenotypes are extremely rare.

Table 3 shows the evaluation of the extended pedigrees of 22 PMS2-deficient cases, only two of which met either Amsterdam I or II criteria for HNPCC. Family histories did not consistently suggest a familial cancer syndrome. It seems that some of the cases in our series represent an HNPCC-like kindred or an attenuated HNPCC-like kindred, whereas other cases may be due to a new mutation dominant disease, low level of penetrance of germ line PMS2 mutations, or may be due to somatic inactivation of PMS2. The published families with homozygous PMS2 mutations have had a very distinct phenotype as described previously, and this phenotype was not seen in this series, perhaps because we were detecting heterozygotes, which maybe much more common.

An analysis of 84 HNPPC and HNPPC-like kindreds without known mutations in MSII, MLH1, and MSH6 failed to identify any pathogenic mutations in the PMS2 gene (38). A possible contribution of PMS2 inactivation to the development of sporadic colon cancers was shown in vitro by Ma et al. (32). In this study, somatic mutations of both alleles resulted in PMS2 gene inactivation in a Vaco481 colon cancer. The PMS2-deficient cell line showed MSI, an elevated HPRT gene mutation rate, and resistance to the cytotoxicity of the alkylator N-methyl-N′-prine-nitronitrosoguanidine. Our report now furthers the likelihood that inactivation of PMS2 may play an important, but limited, role in the development of sporadic MSI-H colorectal cancers.

It is important to note that our analysis was limited by suboptimal fixation of the PMS2 immunostain in six cases which were rendered not interpretable. Whereas the immunohistochemical assay was optimized using controls at each testing center, archived samples were collected from multiple institutions, and inconsistencies in fixation and processing protocols likely contributed to the difficulties encountered in the interpretation of these specimens.

In conclusion, in this series of 2,719 colorectal cancer patients, 535 had MSI-H tumors and in that subgroup, 93% had loss of expression of MLH1, MSH2, and/or MSH6. Of the remaining 38 individuals with MSI-H tumors, 23 of 32 (72%) had selective loss of expression of PMS2. There was an association with young age at cancer diagnosis and right-sided tumors, but this perhaps is accounted for by ascertainment of such cases for clinical testing and research enrollment. Analysis of available pedigrees did not support an extended familial cancer syndrome for most individuals; thus, an attenuated familial phenotype may exist or somatic inactivation of PMS2 may account for a significant proportion of the MSI-H colorectal tumors with selective loss of PMS2 expression by immunohistochemistry. Additional studies will be required to understand the contribution of hereditary alterations in PMS2 in the etiology of colorectal cancers and the mechanisms for inactivation of PMS2 in tumors.

References

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Isolated Loss of PMS2 Expression in Colorectal Cancers: Frequency, Patient Age, and Familial Aggregation

Sharlene Gill, Noralane M. Lindor, Lawrence J. Burgart, et al.


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