Characterization of Mutator Phenotype in Familial Colorectal Cancer Patients Not Fulfilling Amsterdam Criteria


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ABSTRACT

Purpose: Although the mutator phenotype, including genetic and epigenetic alterations of the mismatch repair (MMR) system, seems to be pronounced in familial colorectal cancer, there have been few integrative studies comprising the entire mutator pathway. This study was done to identify the entire mutator pathway determining risk factors in patients with familial colorectal cancer not fulfilling the Amsterdam criteria.

Experimental Design: We consecutively recruited 134 colorectal cancer patients with a family history of accompanying cancers. Patients with hereditary nonpolyposis colorectal cancer meeting the Amsterdam criteria, familial adenomatous polyposis, or those receiving preoperative radiotherapy were excluded. Mutator phenotype was assessed by assaying microsatellite instability (MSI) at 24 markers, hMLH1-promoter methylation, mutations at MMR genes (hMLH1, hMSH2, hMSH6, and hPMS2), and immune staining of MMR proteins (hMLH1, hMSH2, hMSH6, hPMS1, and hPMS2).

Results: Of the 208 cancers in first-degree and/or second-degree relatives of patients, colorectal and gastric cancers (81%) were most common. Of the 134 proband colorectal cancers, 23 (17%) were MSI in high level, and 32 (24%) were MSI in low level. MMR alterations, including known polymorphism and splicing substitution, were identified in eight patients (6%). Twenty-eight tumors with mutator phenotype were further identified by hMLH1-promoter methylation and/or loss of MMR protein expression. In 51 tumors (38%), mutator phenotype was associated with right-sided colon cancer ($P < 0.001$) and younger age at onset ($P = 0.032$), but the number of patients with a mutator phenotype did not differ with respect to inheritance patterns of accompanying cancers, either successive or horizontal transmission ($P = 0.815$). Familial impact value, which differentially associated the degree of relatives with all accompanying cancers, effectively discriminated MSI in high level from microsatellite stable/MSI in low level tumors.

Conclusion: Familial colorectal cancer may be associated with multiple occurrences of colorectal or accompanying cancers inherited by dominant or recessive transmission. MMR gene mutations, however, are less associated with mutator phenotype in familial colorectal cancer.

INTRODUCTION

Hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis are genetically well-defined hereditary colorectal cancers that constitute about 1 to 3% of all colorectal cancers. Another 20% of these cancers are accounted for by colorectal cancer possibly associated with heredity, an ill-defined category of multiple occurrences of colorectal and accompanying cancers in a family. The Amsterdam criteria for HNPCC are strictly defined and exclude most suspect cases of possible heredity-associated tumors. In contrast, the original and revised Bethesda guidelines broaden the disease spectrum by including colorectal cancer families with specific accompanying cancers and clinicopathologic characteristics. These criteria provide important clues for identifying genetic pathways associated with atypical phenotypes of hereditary colorectal cancer. Whereas the Amsterdam criteria regard HNPCC-associated cancers of Amsterdam criteria, such as small bowel, urinary tract and endometrial cancers as equivalent to colorectal cancer, these criteria omit a variety of accompanying cancers, and thus do not consider their frequent occurrence and genetic implication.

HNPCC is caused by germline mutations in any of DNA mismatch repair (MMR) genes, most frequently hMLH1 and hMSH2, and more infrequently, hMSH6, hPMS2, and hPMS1. MMR gene mutations are accompanied by mutator phenotype that is also caused by hMLH1-promoter methylation even if not sufficient for complete gene inactivation. The mutator phenotype generally exhibits microsatellite instability (MSI). Tumors with MSI accumulate hundreds of thousands of somatic clonal mutations, preferentially in simple repeats. MSI frequency seems to be directly proportional to the hereditary basis of a colorectal cancer, with a frequency of 15 to 85% in HNPCC, HNPCC-suspect, and sporadic colorectal cancer. MSI tumors of HNPCC and sporadic colorectal cancers tend to have a proximal location and to be poorly differentiated and mucinous. Therefore, HNPCC and sporadic microsatellite instability in high level (MSI-H) cancers are regarded as familial and sporadic counterparts of the same pathway in tumorigenesis. In...
contrast to HNPCC, the molecular and clinical characteristics of familial colorectal cancer have not been clearly defined. MMR mutation rate is variable among ethnic groups and the number of MMR genes examined. HNPCC and familial colorectal cancers without MMR mutations may be associated with deletions of entire exons, sharing of environmental carcinogens in the presence of HNPCC phenocopies, and molecular mechanisms other than the MMR pathway (7, 8). One transfection study showed functional interactions between weak alleles in the majority of known MMR genes and other genes functioning in DNA replication, i.e., \( \text{POL}32 \) and \( \text{RNR}1 \) (9). Individually, these weak alleles may give rise to a weak mutator phenotype, but when combined, they may give rise to a strong mutator phenotype. The Amsterdam criteria require colorectal cancers to occur in successive generations, thus emphasizing dominant inheritance although neglecting recessive traits in familial colorectal cancer. Segregation analysis of families without MMR mutations has suggested the existence of moderately rare, recessively inherited disease alleles (10, 11). With recessive inheritance, the association between siblings of colorectal cancer would be greater than that between parents and offspring.

Family history is one of the strongest predictors of colorectal cancer. A meta-analysis has shown a 2-fold risk in first-degree relatives of individuals with colorectal cancer, and a 4-fold risk when the relative was diagnosed before age 45 (12). Although MMR mutations are frequently used to diagnose HNPCC, about 50% of individuals suspected of having HNPCC are not confirmed genetically (13). The first step in diagnosing familial colorectal cancer in lower risk families may therefore be MSI assay of colorectal tumors, followed by mutation analysis of patients with MSI tumors. Highly unstable tumors constitute a distinct entity with respect to family history and MMR gene status. \( hMLH1 \)-promoter methylation is another important mechanism of mutator phenotype, especially in sporadic colorectal cancers, with the number of methylated loci shown recently to be closely associated with family history of cancer (13). Immune staining of MMR protein is regarded as a rapid, cost-effective, and accurate method for assessment of mutator phenotype (14). Although strong associations among \( hMLH1 \)-promoter methylation, MSI, and MMR expression have been found, there are discrepancies among molecular and immune staining results. It is apparent that inconsistent results should prompt a search for biological, clinical, and technical explanations for this finding and not be assumed as a wrong one (14). These inconsistent results suggest a need for methods that yield less ambiguous findings.

In our study, familial colorectal cancer was defined broadly to include multiple occurrences of accompanying cancers in a family. We call these cancers familial rather than hereditary because this category of disease is first identified clinically, with as yet undetermined heredity. Although the mutator phenotype seems to be more pronounced in familial colorectal cancer, there have been few integrative studies comprising the entire mutator pathway, i.e., MMR gene mutation and loss of protein expression, MSI, and \( hMLH1 \)-promoter methylation. We therefore assayed this entire mutator pathway in familial colorectal cancer patients not fulfilling Amsterdam criteria, and we determined the association between various mutator phenotypes and the clinicopathologic characteristics including family history of accompanying cancers. These findings should enable us to determine the risk factors and efficient screening tools for the prevention of familial colorectal cancers.

**MATERIALS AND METHODS**

**Patients and Clinicopathologic Finding.** One hundred and thirty-four colorectal cancer patients with a family history of accompanying cancers were consecutively recruited among 1250 colorectal cancer patients between 1999 and 2003 at the Asan Medical Center (Seoul, Korea). Family history was obtained from questionnaire and interview by the physician of colorectal cancer clinic. Both maternal and paternal relatives were interviewed whenever possible. The questionnaire included the family history of cancer in first- and second-degree relatives with regard to their current age, type of cancer, age, and hospital at diagnosis and current status. Other relatives faintly recalled cancer types, which were sometimes confirmed by the medical records from related hospital. Any patients with vague family history were excluded from the enrollment, nevertheless. Histologically identified normal and tumor samples were freshly obtained from each patient. In addition, lymphocyte samples were obtained from each patient and their first-degree relatives. The 134 patients consisted of 71 males with a mean age of 57 years (27–83 years) and 63 females with a mean age of 55 years (23–81 years). Eight cases with HNPCC corresponded to the Amsterdam criteria (either I or II) and 11 cases of familial adenomatous polyposis were excluded. Two patients treated preoperatively with radiotherapy were also eliminated because of possible alteration of tumor DNA. All solid cancers in a family were included as accompanying cancers, except for cancers associated with viral infection, such as primary liver and uterine cervix cancers. By American Joint Committee on Cancer (AJCC) tumor staging (6th ed., 2001), there were 25 patients of stage I, 61 of stage II, 36 of stage III, and 12 of stage IV. Thirty-five tumors were in the right colon, 29 in the left colon, and 70 in the rectum. One hundred and sixteen tumors were well- or moderately differentiated, whereas 18 were poorly differentiated or mucinous. This study was conducted prospectively under the approval of the Institutional Review Board for Human Research.

**Detection of MSI and \( hMLH1 \)-Promoter Methylation.** Primers amplifying respective microsatellite markers (15) were used to determine the MSI by PCR. Twenty-four loci were examined according to National Cancer Institute recommendations and alternative loci as in the International Guidelines (5): \( \text{BAT} 25, \text{BAT}26, \text{BAT}40, \text{BAT}34C4, \text{TGF}\beta\text{RII}, \) and \( \text{ACTC} (635/636) \) for mono-nucleotide repeats; \( \text{DSS}346, \text{D}2\text{S}123, \text{D}1\text{S}250, \text{D}1\text{S}555, \text{D}1\text{S}58, \text{D}1\text{S}61, \) and \( \text{D}1\text{S}64 \) for di-nucleotide repeats; \( \text{D}3\text{S}1029, \text{D}1\text{S}197, \text{D}1\text{S}175, \) and \( \text{D}1\text{S}558 \) for tri-nucleotide repeats; and \( \text{D}5\text{S}107, \text{D}7\text{S}19, \text{D}8\text{S}87, \text{D}1\text{S}153, \text{D}1\text{S}787, \text{D}1\text{S}69, \) and \( \text{D}2\text{S}100 \) for tetra-nucleotide repeats. The PCR products were denatured and subjected to gel electrophoresis and silver staining. MSI was scored positive when there was a definite shift of the PCR product amplified from tumor DNA compared with that from normal tissue DNA. All MSI-positive loci were confirmed by duplicate assays. MSI-H (i.e., a high level of microsatellite instability) was defined when \( \geq 40\% \) of markers showed MSI; MSI-L (i.e., a low level of micro-
satellite instability) when <40% of markers showed MSI; and microsatellite stable (MSS) was defined when none of the assayed markers showed MSI.

Methylation of the promoter region in hMLH1 was determined by methylation-specific PCR, as described previously (16). Methylation-specific PCR distinguishes unmethylated from methylated alleles based on sequence alterations produced by bisulfite treatment of DNA, which converts unmethylated but not methylated cytosine to uracil, followed by PCR that used primers specific to either methylated or unmethylated DNA. Primer sequences were selected covering the upstream region of the hMLH1 promoter, i.e., nucleotide −716 to nucleotide −602 (17). Primers for unmethylated DNA were 5′-TTTTGATGTA-GATGTTATTAGGGTTG-3′ (sense) and 5′-ACCACCTC-CATCATAACTCCCCACA-3′ (antisense), whereas those for methylated DNA were 5′-ACGTAGACGTATTAGGGTGCA-3′ (sense) and 5′-CCTCATCGTAACTCCCCGGC-3′ (antisense). Briefly, 1 μg of genomic DNA was denatured with NaOH and treated with sodium bisulfite. Ten μl of each PCR reaction product were loaded directly onto nondenaturing 6% polyacrylamide gels, which were stained with ethidium bromide and visualized under UV illumination. DNA from the colon cancer cell line SW48, which is completely methylated at the hMLH1-promoter region, was used as a positive control, whereas DNA from normal tissue was used as a negative control.

Single-Strand Conformation Polymorphism-PCR and Sequencing. Single-strand conformation polymorphism-PCR was done on all samples in which MSI-H or loss of any MMR protein expression had been detected. Primers were selected covering all exonic and flanking intronic sequences of hMLH1, hMSH2, hMSH6, and hPMS2 (18–20). Each 50 μl of reaction mixture contained approximately 200 ng of template DNA, standard PCR buffer (Promega, Madison, WI), 2 mmol/L Mg2+, 0.5 mmol/L of each deoxynucleoside triphosphate, 0.4 mmol/L of each specific oligonucleotide primer, and 1 μL Taq polymerase. The amplification protocol consisted of an initial denaturation at 94°C for 4 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at the predetermined optimal temperature for 1 minute, and extension at 72°C for 1 minute with a final extension at 72°C for 10 minutes. PCR products were heated to 90°C for 5 minutes and quenched on ice. Electrophoresis was done in 10% non-denaturing polyacrylamide gels at 20 mA for 18 hours, and DNA bands were detected by silver staining. Aberrant bands identified on single-strand conformation polymorphism-PCR were sequenced on an ABI 377 prism sequencer (Perkin-Elmer, Norwalk, CT), with repeated sequencing used to verify mutations for both forward and reverse primers.

Immunohistochemical Staining. Paraffin-embedded tissue cores of tumors were collected and used for the construction of tissue microarray. A precision instrument (Beecher Instrument Inc., Sun Prairie, WI) was used to array triplicate 1-mm tissue cores onto recipient paraffin blocks. A DAKO LSAB kit (DAKO, Carpinteria, CA) was used to subject tissue array blocks containing core cylinders to immune staining based on the labeled streptavidin-biotin method. Briefly, slides were deparaffinized with xylene and dehydrated in graded ethanol. The antigen was retrieved by dipping into boiled citrate buffer [10 mmol/L (pH = 6)] for 10 minutes in a microwave oven. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide for 10 minutes. The slides were incubated for 2 hours with appropriately characterized and diluted monoclonal antibodies to hMLH1 (G168–15), hMSH2 (G219–1129, BD PharMingen, San Diego, CA), MSH6 (Serotec Laboratories, Kidlington, United Kingdom), hPMS1 (sc-615), PMS2 (sc-618, Santa Cruz Biotechnology Inc., Santa Cruz, CA), and P53 (DO-7, DAKO, Glostrup, Denmark). The slides were incubated for 10 minutes each with biotinylated-linked secondary antibody and peroxidase-labeled streptavidine, and subsequently with substrate-chromogen solution. All slides were counterstained with hematoxylin solution. For all samples with negative immune staining on a tissue microarray, the whole tissue sections were repeatedly assayed. The percentage of cells positively stained was divided into two grades for MMR proteins (i.e., − for ≤10% and + for >10% nuclear staining) and into four grades for P53 (<10%, 10–30%, 30–50%, and ≥50%; ref. 15).

Statistical Methods. An Altman’s nonogram, assuming a putative MSI-H incidence as 15–40%, was used to determine the sample size to obtain the 80% power to detect MSI-H in familial colorectal cancer. Cross-table analysis that used Fisher’s exact test compared the respective mutator phenotype with MSI, hMLH1-promoter methylation, protein expression, and clinicopathologic variables. Multivariate analysis that used logistic regression verified all variables showing statistical significance. Unpaired Student’s t test tested familial impact value on mutator phenotype. The significance level was set at 5% for each analysis, and an IBM-PC with SPSS software (ver.11, SPSS Inc., Chicago, IL) performed all calculations.

RESULTS

Familial Characteristics. There were 208 colorectal and other cancers present in the first-degree and/or second-degree relatives of 134 colorectal cancer patients in this study. The 208 cancers consisted of 89 colorectal cancers, 79 gastric cancers, 5 HNPPC-associated cancers (small bowel, urinary tract, and endometrial cancers), 18 other digestive cancers (biliary, pancreatic, and esophageal cancers), and 17 other common cancers (lung, breast, and ovarian cancers; Fig. 1). Among the 134 probands, 98 (73%) had at least one affected first-degree relative, 17 (13%) had at least one affected second-degree relative, and 18 (13%) had at least one affected both first- and second-degree relatives. Approximately one-third of cancers was diagnosed before age 50.

MSI Status and hMLH1-Promoter Methylation. Of the 134 colorectal tumors, 23 (17%) were MSI-H and 32 (24%) were MSI-L. The mean number of markers showing instability was 16 (range, 10–21) and 2 (range, 1–4), respectively. The MSI rate of respective markers did not differ in nucleotide repeat types, ranging from 28 to 36%. Seventeen of 24 (71%) markers showed MSI in >90% of MSI-H tumors (Fig. 2). Five National Cancer Institute-recommended markers accurately identified MSI-H tumors, but they missed 25 of 34 MSI-L tumors. We found that BAT25, BAT26, and D17S250 accurately identified MSI-H when two or more
showed MSI. Twenty-two of 64 (34%) colon cancers presented as MSI-H, compared with only 1 of 70 (1.4%) rectal cancers did ($P < 0.001$). Younger age at onset, more accompanying cancers, right-sided colon cancer, infiltrative growth, and poorly differentiated or mucinous colorectal cancer were each closely associated with MSI-H (Table 1, $P < 0.001$–0.024). Multivariate logistic regression showed that the number of accompanying cancers and right-sided colon cancers were significantly greater in patients with MSI-H tumors than in those with MSS or MSI-L tumors ($P < 0.001$ and = 0.009, respectively). MSI-H prevalence was two and one-half times greater in patients with two or more colorectal cancers in their family than in those with one colorectal cancer (23.9% versus 9.5%, $P = 0.038$). The number of patients with MSI-H tumor did not differ with respect to inheritance patterns of accompanying cancers, i.e., either successive or horizontal transmission (15 of 83 versus 8 of 51, $P = 0.816$). Similarly, the number of patients with MSI-H tumors did not differ between families of successive and horizontal generations affected with colorectal cancer or HNPCC-associated cancers (4 of 22 versus 8 of 36, $P = 1$). Forty tumors (30%) showed MSI at loci for colorectal cancer-associated tumor suppressor genes, with 28 showing MSI at $hMLH1$, $APC$, and $CTNNB1$, and 33 at the other genes ($SMAD4$, $DCC$, $MCC$, $PSEN$, $P53$). Nineteen tumors (14%) showed MSI concurrently in these three categories of tumor suppressor genes.

$hMLH1$-promoter methylation was identified in 30 patients (22%). The mean rate of microsatellite instability at 24 markers in these tumors was 23% (range, 10–33%), compared with 10% (range, 5–14%) in tumors without $hMLH1$-promoter methylation. Thirteen markers (BAT25, BAT26, BAT40, D20S200, TGFBR2, DSS107, D13S153, D17S588, D19S55, D18S611, D18S64, and BAT32C4) were accompanied prominently by $hMLH1$-promoter methylation ($P < 0.001$–0.041). Ten of 23 MSI-H tumors (44%) showed $hMLH1$-promoter methylation, compared with 20 of 111 MSS/MSI-L tumors (18%; $P = 0.008$). Seven of the 13 MSI-H tumors (54%) without methylation had MMR gene alterations. Among clinicopathologic parameters, $hMLH1$-promoter methylation closely correlated with right-sided colon cancer by multivariate analysis ($P = 0.007$).
MMR Gene Alterations. Five different alterations were found in eight patients including two in hMSH2 and one each in hMLH1, hMSH6, and hPMS2 (Table 2). Among six of them, the MMR alteration was identical among patients and first-degree relatives, which did not co-segregate with colorectal or associated cancers in the relatives. Two patients concurrently carried two alterations in hMSH2/hMLH1 and hMSH2/hPMS2. Each of these patients had two siblings with colon cancer. Two novel mutations, i.e., one frameshift and one intronic alteration, were identified (1039ins TT in hMSH2 and gIVS1896 + 36 C→T in hMLH1). The other three alterations included one intronic base substitution and two missense mutations (gIVS211 + 9 C→G in hMSH2, G39E in hMSH6, and P470S in hPMS2). All eight patients with MMR alterations occurred in MSHI-H tumors. All but one patient (case no. 25) with MMR alterations showed a loss in expression of at least one MMR protein. All three patients with novel mutations and five of six patients with other alterations had two or more first- or second-degree relatives with colorectal cancer.

MMR Protein Expression. The mean false-negative detection rate was 8.8% (range, 6.8–11.2%) on tissue microarray, which did not differ with respective MMR proteins. hMLH1 expression was closely correlated with expression of hPMS1 and hPMS2 expressions, whereas hMSH2 expression was correlated with hMSH6 expression (P < 0.001). A significant correlation was also observed between hMLH1 and hMSH2 expression (P = 0.017). MSI status was also closely associated with MMR protein expression (Fig. 3). Twenty of 23 MSHI-H tumors (87%) showed absence of at least one MMR protein expression, compared with 11 of 111 MSS or MSHI-L tumors (10%; P < 0.001). Twelve of 30 tumors (40%) with hMLH1-promoter methylation did not express any one of MMR proteins, whereas 85 of 104 tumors (82%) without hMLH1-promoter methylation expressed five of the MMR proteins examined (P = 0.025). The accuracy rate was 80% (107 of 134) when both hMLH1 and hMSH2 protein expressions were assayed. Accuracy was highest when five MMR proteins were evaluated (114 of 134, 85%) but was almost as high when hPMS2 and hMSH6 were added to hMLH1 and hMSH2 (113 of 134, 84%). Otherwise, the sensitivity for the evaluation of mutator phenotype was 100%, regardless of combinations of immune staining such as hMLH1 and hMSH2 proteins alone or with the addition of other MMR proteins. The number of accompanying cancers, right-sided colon cancers and younger age at onset colorectal cancers each showed higher association with loss of MMR protein expression than with MMR expression (P < 0.001–0.035). Right-sided colon cancers positively correlated with loss of any MMR protein expression, except for hPMS1, by multivariate analysis (P < 0.001).

### Table 1 Comparison of clinicopathologic parameters in MSI-H and MSI-L/MSS colorectal cancers

<table>
<thead>
<tr>
<th></th>
<th>MSI-H (n = 23)</th>
<th>MSI-L/MSS (n = 111)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, M/F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age, &lt;50/&gt;50</td>
<td>10/13</td>
<td>61/50</td>
<td>0.363</td>
</tr>
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<td>Affected patients, 1st-/2nd-degree relative*</td>
<td>19/4</td>
<td>98/13</td>
<td>0.493</td>
</tr>
<tr>
<td>Inheritance, successive/horizontal†</td>
<td>15/8</td>
<td>67/44</td>
<td>0.815</td>
</tr>
<tr>
<td>Type of accompanying cancer, HNPCC-associated/other cancers‡</td>
<td>17/6</td>
<td>58/53</td>
<td>0.067</td>
</tr>
<tr>
<td>No. of accompanying cancer, &lt;2/&gt;2</td>
<td>7/16</td>
<td>76/35</td>
<td>0.001</td>
</tr>
<tr>
<td>Tumor location, right-/left-sided</td>
<td>20/3</td>
<td>15/96</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AJCC stage, I/II/III/IV</td>
<td>1/15/5/2</td>
<td>24/46/31/10</td>
<td>0.128</td>
</tr>
<tr>
<td>Growth type, expanding/infiltrative</td>
<td>17/6</td>
<td>101/9</td>
<td>0.024</td>
</tr>
<tr>
<td>Differentiation, WD, MD/PD, mucinous</td>
<td>16/7</td>
<td>100/11</td>
<td>0.016</td>
</tr>
<tr>
<td>Concurrent adenomas, yes/no</td>
<td>12/11</td>
<td>48/63</td>
<td>0.493</td>
</tr>
</tbody>
</table>

* Affected patients with colorectal and accompanying cancers in the pedigree.
† Inheritance pattern of colorectal and accompanying cancers in a family.
‡ HNPCC-associated cancers including small bowel, endometrial and urinary tract cancers; other cancers, other solid cancers exclusive of hematologic and virus-associated cancers.

NOTE. All parameters were verified by Fisher’s exact test except for AJCC stage, which was verified by Pearson’s χ² test.

**Abbreviations:** WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated.

### Table 2 Mismatch repair gene alterations in familial colorectal cancer patients

<table>
<thead>
<tr>
<th>ID</th>
<th>Genotype</th>
<th>MMR</th>
<th>Exon/intron</th>
<th>Base change</th>
<th>Consequence</th>
<th>Mutation in relatives</th>
</tr>
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<tbody>
<tr>
<td>94, 124</td>
<td>Homo</td>
<td>hMLH1</td>
<td>gIVS1896 + 36</td>
<td>C→T</td>
<td>NA</td>
<td>94/son (+), 124/daughter (+)</td>
</tr>
<tr>
<td>5</td>
<td>Hetero</td>
<td>hMLH1</td>
<td>gIVS211 + 9</td>
<td>C→G</td>
<td>Stop</td>
<td>10,94/son (−), 10, 94/daughter (+)</td>
</tr>
<tr>
<td>10, 94</td>
<td>Homo</td>
<td>hMLH1</td>
<td>116G→A</td>
<td>G39E</td>
<td>NA</td>
<td>91/sister (−), 91/sister (+)</td>
</tr>
<tr>
<td>19, 25, 91</td>
<td>Hetero</td>
<td>hMLH1</td>
<td>11408C→T</td>
<td>P470S</td>
<td></td>
<td>10/son (+), 75/daughter (+)</td>
</tr>
<tr>
<td>10, 75</td>
<td>10 (Homo), 75 (Hetero)</td>
<td>hPMS2</td>
<td>11</td>
<td></td>
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</table>

Abbreviation: NA, not assessed.
* (+), same mutation as in the patient.
† (−), no mutation.
Correlation of Mutator Phenotypes with Clinicopathologic Parameters. Mutator phenotype, including genetic and epigenetic alterations of MMR system, was defined when any tumor in which MSI-H, hMLH1-promoter methylation, or loss of MMR protein expression was identified. Fifty-one patients (38%) exhibited mutator phenotype. There were three times as many colon cancers as rectal cancers in patients with mutator phenotypes (38 to 64 versus 13 to 70, \( P < 0.001 \)). Right-sided colon cancer and younger age at onset colorectal cancer were closely associated with mutator phenotypes (Table 3, \( P < 0.001 \) and \( P < 0.032 \), respectively). The number of patients with mutator phenotype, however, did not differ with respect to inheritance patterns of accompanying cancers, either in successive or horizontal generations (\( P = 0.815 \)). Multivariate analysis showed that right-sided colon cancers were significantly higher in patients with mutator phenotypes than those without them (\( P < 0.001 \)). Mutator phenotype was more frequently found in tumors without than those with altered P53 expression (Table 3; \( P = 0.048 \)). Among chromosome 17 markers, altered P53 expression was closely associated with BAT34C4 and D17S250 (\( P = 0.022 \) and 0.04, respectively) but not with D17S588 and D17S787. Poorly differentiated or mucinous tumors occurred over three times more frequently in tumors without than those with altered P53 expression (\( P = 0.022 \) by multivariate analysis).

Familial Impact Values Regarding Mutator Phenotype. We defined the familial impact value (FIVK), calculated as the sum for each patient of colorectal and accompanying cancers in each family multiplied by the relative degree (1 for each first-degree relative and 0.5 for each second-degree relative). FIVK was significantly higher in patients with MSI-H than MSS/MSI-L tumors when any categories of accompanying cancers were included (Fig. 4A; \( P = 0.004 – 0.013 \)). When FIVK was calibrated by type of cancer (1 for colorectal and HNPCC-associate, and 0.5 for other accompanying cancers).

### Table 3  Comparison of clinicopathologic parameters regarding mutator phenotypes

<table>
<thead>
<tr>
<th></th>
<th>Mutator (+) (( n = 51 ))</th>
<th>Mutator (-) (( n = 83 ))</th>
<th>( P )</th>
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<tbody>
<tr>
<td>Sex, M/F</td>
<td>22/29</td>
<td>49/34</td>
<td>0.078</td>
</tr>
<tr>
<td>Age, ( \leq 50/&gt;50 )</td>
<td>21/30</td>
<td>19/64</td>
<td>0.032</td>
</tr>
<tr>
<td>Affected patients, 1st-/2nd-degree relative*</td>
<td>44/7</td>
<td>73/10</td>
<td>0.794</td>
</tr>
<tr>
<td>Inheritance, successive/horizontal†</td>
<td>34/17</td>
<td>48/35</td>
<td>0.815</td>
</tr>
<tr>
<td>Type of accompanying cancer, HNPCC-associated/other cancers‡</td>
<td>30/21</td>
<td>45/38</td>
<td>0.72</td>
</tr>
<tr>
<td>No. of accompanying cancers, ( \leq 2/&gt;2 )</td>
<td>28/23</td>
<td>55/28</td>
<td>0.204</td>
</tr>
<tr>
<td>Tumor location, right-/left-sided</td>
<td>26/25</td>
<td>9/74</td>
<td>&lt;0.001</td>
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<tr>
<td>AJCC stage, II/III/IV</td>
<td>7/22/14/3</td>
<td>18/34/22/9</td>
<td>0.4</td>
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<tr>
<td>Growth type, expanding/infiltrative</td>
<td>43/8</td>
<td>75/7</td>
<td>0.262</td>
</tr>
<tr>
<td>Differentiation, WD, MD/PD, mucinous</td>
<td>42/9</td>
<td>74/9</td>
<td>0.302</td>
</tr>
<tr>
<td>Concurrent adenomas, yes/no</td>
<td>24/27</td>
<td>36/47</td>
<td>0.722</td>
</tr>
<tr>
<td>Altered P53 expression, yes/no</td>
<td>23/28</td>
<td>51/32</td>
<td>0.048</td>
</tr>
</tbody>
</table>

NOTE. All parameters were verified by Fisher’s exact test except for AJCC, which was verified by Pearson’s \( \chi^2 \) test.

Abbreviation: Mutator (+), any of MSI-H, hMLH1 promoter methylation, and loss of MMR protein expression; mutator (-), no mutator phenotype; WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated.

* Affected patients with colorectal and accompanying cancers in the pedigree.
† Inheritance pattern of colorectal and accompanying cancers in a family.
‡ HNPCC-associated cancers including small bowel, endometrial and urinary tract cancers; other cancers, other solid cancers exclusive of hematologic and virus-associated cancers.
paching cancers; FIV<sup>K+AC</sup>), the difference between MSI-H and MSS/MSI-L was more prominent (Fig. 4B). In MSI-H tumors, the mean FIV<sup>K+AC</sup> in patients without hMLH1-promoter methylation was significantly greater than those with methylation (2.5 ± 0.6 versus 1.8 ± 0.6, P = 0.001).

**DISCUSSION**

Comparative genomic study that used microarray has shown that MSI-H exerts a dominant impact on global molecular phenotype in colorectal cancer (21). We have shown here that only 17% of tumors were MSI-H, an incidence similar to the 12 to 18% prevalence in sporadic colorectal cancer (3, 22) and significantly lower than reported in HNPCC and patients with suspected HNPCC (5, 23, 24). BAT26 was used as the basis of a study on a limited assay that showed MSI-H rate as 9% in the 230 unsampled cases of Korean sporadic colorectal cancer (25). Approximately 70% of 24 markers in our study accurately identified MSI-H, indicating that these markers could be applied to identifying these tumors. In the MSI-H tumors in our study, the mean MSI rate of 24 markers was 67% (range, 42% to 88%), which exceeded the suggested criteria of 20 to 40% (5, 26). One study reported a discrepancy rate of 38% between the five National Cancer Institute-recommended markers and 35 additional markers (27). We found that the five reference markers completely identified MSI-H in our study, but these markers missed >75% of MSI-L tumors, thus indicating that the MSI rates in MSI-H and MSI-L tumors were clearly distinct. We also found that most MSI-L tumors were unstable at one or two marker. Most non-MSI-H tumors are known to exhibit extremely low frequencies of MSI when a large number of microsatellite loci are tested (28). In our study, MSI-L and MSS tumors did not differ with respect to clinicopathologic characteristics as reported in the other investigations, to elucidate phenotypic and biological similarities between them (5, 14).

Approximately 500 mutations in hMLH1, hMSH2, and hMSH6 have been reported to date, with >40% of these mutations being missense or splicing variants. One patient with hMSH2 frameshift mutation (1039ins TT) may have HNPCC, but three mutations in our study that had been reported previously suggested as polymorphisms (gIVS211 + 9 C→G in hMSH2, G39E in hMSH6, and P470S in hPMS2). Although these polymorphisms and the other splicing defect were identified without disease co-segregation in a limited relatives of our patients, the hereditary aspects of these alterations remains to be determined by follow-up family studies. Some missense mutations may be associated with a familial predisposition to colorectal cancer, whereas others may be non-functioning polymorphisms, as shown in population-based and functional studies (24, 29). MMR genes and other genes involved in DNA replication may cause a strong mutator phenotype when they are combined with each other, as in the two patients of our study, although each of these individual alleles may give rise to a weak mutator phenotype (30). Mutation prevalence in our study was 6%, including polymorphism and splicing substitution. This figure, together with low MSI-H rate, seems to indicate that MMR mutations induce a lower selection pressure in most of familial colorectal cancer not fulfilling Amsterdam criteria. HNPCC family not complying with Amsterdam criteria generally have frequency of MMR mutations 8 to 50% (2, 24, 31, 32). There may be a few mutations or genomic rearrangements that have not been identified by single strand conformation polymorphism-PCR. One investigation showed 14 genomic rearrangements in hMSH2 and hMLH1 in 55 HNPCC and HNPCC-suspect families, whereas only a few genomic rearrangements, even in hMSH2, were identified in families that did not sufficiently meet Amsterdam criteria (8, 33).

In contrast to one study, which found that methylation of upstream region (nucleotide −716–nucleotide −602) of hMLH1 promoter did not correlate with loss of hMLH1 expres-

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**Fig. 4** Association of FIV<sup>K</sup> with relative degree and FIV<sup>K+AC</sup> with relative degree and accompanying cancers. A, colorectal and HNPCC-associated cancers (P value between MSI-H (●) and MSI-L/MSS (○) tumors, 0.006 for FIV<sup>K</sup> and 0.003 for FIV<sup>K+AC</sup>); B, “A” plus digestive cancers (0.004 for FIV<sup>K</sup>; <0.001 for FIV<sup>K+AC</sup>); C, “A” plus other digestive cancers (0.013 for FIV<sup>K</sup>; 0.004 for FIV<sup>K+AC</sup>); D, “A” plus other common cancers (0.004 for FIV<sup>K</sup>; 0.001 for FIV<sup>K+AC</sup>); E, all accompanying cancers (0.007 for FIV<sup>K</sup>; 0.001 for FIV<sup>K+AC</sup>).
sion (34), we observed a close correlation between the two. We found that the sensitivity of hMLH1-promoter methylation was 40% in predicting MMR protein expression and that 17 of 18 false-positives occurred in MSI-L/MSS tumors. It is likely therefore that methylation of the CpG sites is important but not sufficient for gene inactivation. Methylation of the hMLH1 promoter has been found to initiate MSI, leading to a temporal window during which some tumors have not yet accumulated large numbers of MSI alterations and showing proficient methylation in MSI-L (35). Six of 23 MSI-H tumors carried neither methylation nor MMR mutation in our study. In addition, eight MSI-H tumors were reported to show no hMLH1-promoter methylation among 47 colorectal cancer patients without MMR mutation (13). The possibility of methylation of the other MMR genes or a large deletion/translocation mutations cannot be excluded, however. Because hMLH1-promoter methylation was associated with MSI at specific markers in our study, methylation seemed to cause MSI in a locus-specific manner.

Tissue microarray technology has been validated in colorectal carcinoma as a useful tool for rapid analysis of a large number of samples with molecular alterations. These must safely be accompanied by conventional whole sections, however, to reduce the probability of false-negatives in cases of loss of protein expression. In our study, hMLH1 expression closely correlated with hPMS1 and hPMS2, whereas hMSH2 expression could be correlated individually with expression of hMLH1 and hMSH6. Interaction among hMLH1, hMLH3, and hPMS1 have been reported, and hPMS2 may be degraded in the absence of its binding partner hMLH1 (36). In contrast, the hMSH2-hMSH6 and hMSH2-hMSH3 complexes are known to recognize DNA mispairs and insertion/deletion loops (29, 37). Concurrent loss of hMLH1 and hMSH2 might reflect fulminating mutator phenotype. In our study, MMR protein expression was closely correlated with MSI status, showing sensitivity and specificity as 87 and 90%, respectively. Although a few studies have raised a deficient sensitivity of immune staining in the diagnosis of MSI, most studies validated immune staining as an accurate assay for MSI (38). MMR-deficient tumors are usually detected by staining for hMLH1 and hMSH2, with occasional addition of hMSH6, and these methods have a sensitivity of 88 to 100% and a specificity of 95 to 100% (13, 38). Some MSI-H tumors may show normal expression of hMLH1 and hMSH2, possibly because of immunologic reactivity to functionally inactive mutant protein, presence of the wild-type allele, or hMSH6 mutation.

In our study, >70% of accompanying cancers were colorectal and gastric, with the rest consisting of HNPCC-associated and other digestive and common cancers. The HNPCC-associated cancers, (i.e., small bowel, urinary tract, and endometrial cancers) made up at most 2% of the accompanying cancers in our patients. Gastric cancer is the most prevalent extracolonic malignancy in Korean and Japanese patients with HNPCC and in those with multiple primary cancers accompanying colorectal cancer (2). Many studies, including International Collaborative Group-HNPPC, have found that gastric, ovarian, urinary tract, small bowel, hepatobiliary, and skin cancers are the cancers associated with HNPCC, with brain tumors and prostate occasionally reported in HNPCC-kindred (32, 39). In our study, the familial impact value of MSI status was remarkably great when all accompanying cancers were included together with colorectal and HNPCC-associated cancers, suggesting that most solid cancers should be considered as associated cancers in patients with familial colorectal cancer. Tumors with mutator phenotype closely correlated with younger age at onset. Colorectal cancer diagnosis at an early age seems to be a universal feature of either HNPCC or HNPCC-suspect kindreds.

In our study, the number of patients with mutator phenotype did not differ with respect to inheritance pattern of accompanying cancers, i.e., successive or horizontal generation. In other words, associations among horizontal generations would be similar to those among successive generations in familial colorectal cancer. Segregation analysis of families without MMR mutations has suggested the existence of a moderately rare, recessively inherited disease allele (10, 11, 30). In our study, two patients concurrently carrying two MMR base substitutions each had two siblings, but no parents, who had colon cancer. A similar inheritance pattern has been observed in two families with two concurrent MMR missense mutations, suggesting recessive rather than dominant inheritance (30).

Of our familial colorectal cancer patients, 38% had a mutator phenotype. In hereditary colorectal cancer, molecular genetic mechanisms other than the MMR pathway suggest the possibility of shared environmental carcinogens and the presence of HNPCC phenocopies exclusive of unidentified MMR alterations (7, 8). In agreement with previous studies, we found that tumors with mutator phenotype closely correlated with right-sided colon cancers, as well as with poorly differentiated or mucinous colorectal cancers (6, 38, 40). The differences in MSI rate among cancers may be attributable to various tumor growth patterns, such that a greater number of divisions would be associated with stochastic accumulation of a higher MSI frequency at normal mutation rate (28). Unexpectedly, we found that the rate of MSI-H tumors of rectal cancers (1 in 70) was even lower than the rate reported in 107 HNPCC-suspected patients (1 in 22), suggesting that rectal cancers might be excluded from HNPCC (24, 40). Other researchers, however, have reported a higher incidence (up to 56%) of rectal or rectosigmoid cancers in HNPCC (41). In our study, MSI was implicated in the aberration of as many as 30% of the tumor suppressor genes associated with colorectal tumorigenesis. In some colorectal cancers, the mutator phenotype targets tumor suppressor genes related to early stages of tumorigenesis, such as APC. Studies on concurrent HNPCC adenomas suggested an accelerated adenoma-carcinoma sequence in HNPCC, with MSI rates of 24% in benign adenomas and 54% in adenomas with malignancy (42). We also found an inverse relationship between altered P53 expression and mutator phenotype or poorly differentiated or mucinous, suggesting that the mutator phenotype and histologic properties may occur prior to P53 alterations. Alternatively, these tumors may not undergo P53 alterations. P53 mutations have been correlated with left-sided colorectal cancer, in contrast to MSI, which is correlated with right-sided colon cancer (43). These results suggest the existence of two different pathways, with right- and left-sided colon cancer correlated with mutator phenotype and P53 mutation, respectively.

Familial impact value (FlvK + AC) in our study assumed that the number of alleles shared between the subjects varied by the degree of relationship and the differential association of colorectal cancer and other accompanying cancers in a ancestry.
This value, which showed a significant difference between MSI-H and MSI-L/MSS tumors, could be determined by a simple calculation, suggesting it may be used efficiently as a risk index in familial colorectal cancer. Therefore, screening by family history would be the primary approach identifying the families at risk. The current standard for assessing DNA mismatch pair competency is molecular MSI testing (14). In our study, MSI-H was identified by the combination of BAT25, BAT26, and D17S250, indicating that these loci are the first choice among any reference panel. Evaluation of expression of five MMR proteins, together with hMLH1-promoter methylation, enabled us to identify 21% of non-MSI-H tumors with mutator phenotypes. In conclusion, familial colorectal cancer can be comprehensively explained as multiple occurrences of colorectal and accompanying cancers, by dominant or recessive transmission. MMR gene mutation is infrequent, with >60% of familial colorectal cancer not exhibiting a mutator phenotype, indicating that a molecular genetic mechanism, other than MMR ancestry, remains to be identified in familial colorectal cancer. Because our study was limited to patients with a Korean ancestry, a larger cohort study including different races is needed to verify the clinical and genetic characteristics of familial colorectal cancer.

REFERENCES

Characterization of Mutator Phenotype in Familial Colorectal Cancer Patients Not Fulfilling Amsterdam Criteria


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