Features of Colorectal Cancers with High-Level Microsatellite Instability Occurring in Familial and Sporadic Settings

Parallel Pathways of Tumorigenesis

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High-level microsatellite instability (MSI-H) is demonstrated in 10 to 15% of sporadic colorectal cancers and in most cancers presenting in the inherited condition hereditary nonpolyposis colorectal cancer (HNPCC). Distinction between these categories of MSI-H cancer is of clinical importance and the aim of this study was to assess clinical, pathological, and molecular features that might be discriminatory. One hundred and twelve MSI-H colorectal cancers from families fulfilling the Bethesda criteria were compared with 57 sporadic MSI-H colorectal cancers. HNPCC cancers presented at a lower age ($P < 0.001$) with no sporadic MSI-H cancer being diagnosed before the age of 57 years. MSI was less extensive in HNPCC cancers with 72% microsatellite markers showing band shifts compared with 87% in sporadic tumors ($P < 0.001$). Absent immunostaining for hMSH2 was only found in HNPCC tumors. Methylation of *bMLH1* was observed in 87% of sporadic cancers but also in 55% of HNPCC tumors that showed loss of expression of hMLH1 ($P = 0.02$). HNPCC cancers were more frequently characterized by aberrant β -catenin immunostaining as evidenced by nuclear

positivity ($P < 0.001$). Aberrant p53 immunostaining was infrequent in both groups. There were no differences with respect to 5q loss of heterozygosity or codon 12 *K-ras* mutation, which were infrequent in both groups. Sporadic MSI-H cancers were more frequently heterogeneous ($P < 0.001$), poorly differentiated ($P = 0.02$), mucinous ($P = 0.02$), and proximally located ($P = 0.04$) than HNPCC tumors. In sporadic MSI-H cancers, contiguous adenomas were likely to be serrated whereas traditional adenomas were dominant in HNPCC. Lymphocytic infiltration was more pronounced in HNPCC but the results did not reach statistical significance. Overall, HNPCC cancers were more like common colorectal cancer in terms of morphology and expression of β -catenin whereas sporadic MSI-H cancers displayed features consistent with a different morphogenesis. No individual feature was discriminatory for all HNPCC cancers. However, a model based on four features was able to classify 94.5% of tumors as sporadic or HNPCC. The finding of multiple differences between sporadic and familial MSI-H colorectal cancer with respect to both genotype and phenotype is consistent with tumorigenesis through parallel evolutionary pathways and emphasizes the importance of studying the two groups separately. (*Am J Pathol* 2001, 159:2107–2116)

Colorectal cancer is a condition associated with considerable underlying genetic heterogeneity. It can be classified according to the level of microsatellite instability exhibited by the tumor, into MSI-H (high-level microsatellite instability), MSI-L (low-level microsatellite instability),

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ty), and MSS (microsatellite stable).^{1,2} MSS cancers constitute the majority of colorectal tumors (70 to 80%) and are seen in the sporadic setting as well as in the hereditary disorder familial adenomatous polyposis. MSI-L cancers account for a further 10 to 15% of colorectal cancers and are defined by the presence of limited instability in higher order repeat sequences. Until recently, the etiology of the MSI-L phenotype had been uncertain. It has now been shown that a subset of these tumors have an underlying defect in O-6-methylguanine methyltransferase, a molecule responsible for removing methyl adducts from guanosine residues. When O-6-methylguanine methyltransferase is silenced by methylation, the subsequent generation of methylguanine adducts may overwhelm the DNA mismatch repair system and result in the development of small mutations.^{3,4}

MSI-H cancers comprise the balance of colorectal adenocarcinomas, representing 10 to 15% of sporadic colorectal cancers and virtually all cancers occurring in the familial syndrome hereditary nonpolyposis colon cancer (HNPCC). MSI-H cancers are defined by the presence of MSI in mononucleotide repeats as well as higher order repeats and can also be distinguished from MSI-L cancers by a set of clinical and pathological features that includes proximal location, mucinous histology, poor differentiation and the presence of tumor-infiltrating lymphocytes, and the demonstration of mismatch repair gene deficiency, most of which have diagnostic utility.² After the recognition of MSI-H cancers in 1993, HNPCC and sporadic MSI-H cancers came to be regarded as the familial and sporadic counterparts of the same pathway of tumorigenesis. It may therefore be difficult to distinguish between MSI-H cancers occurring as part of the HNPCC syndrome and sporadic MSI-H cancers, especially when the patient is of intermediate age and is uncertain of family history details.

Family history is considered the most useful indicator of HNPCC in individual patients in the clinical setting. Counterintuitively, no association between MSI-H status and a family history of colorectal cancer (CRC) is seen at the population level.⁵⁻⁷ It has therefore been proposed that MSI status is not a useful clinical indicator of genetic predisposition.⁷ The lack of association of MSI-H phenotype and family history is readily explained. First, the frequency of CRC because of HNPCC is low. Indeed, population-based surveys in high-risk areas for CRC suggest a figure as low as 1%, indicating that only ~7% of MSI-H cancers would occur in the context of HNPCC.⁸ Second, a proportion of individuals with HNPCC will lack a strong family history.^{9,10} Finally, up to 17% of patients with CRC will have an affected first-degree relative, yet few of these family clusters of CRC will be because of HNPCC.^{11,12} Endoscopic surveillance of patients with HNPCC reduces mortality due to colorectal cancer.¹³ With this fact in mind, criteria for instituting selective MSI testing that reflect clinical (notably family history and young age at onset of malignancy) and pathological features indicative of HNPCC have been proposed.¹⁴ The availability of monoclonal antibodies to the DNA mismatch repair proteins hMLH1, hMSH2, hPMS2, and hMSH6 not only simplifies testing for a DNA repair defi-

ciency but also pinpoints the underlying causative gene. Nevertheless, selective testing will miss instances of HNPCC presenting at older than the age of 45 years and lacking a strong family history. Some of the cancers presenting in these individuals may be suspected as being MSI-H on histopathological grounds, because proximal mucinous or poorly differentiated cancers and cancers showing intraepithelial or tumor-infiltrating lymphocytes or a Crohn-like reaction are more likely to be MSI-H.^{2,15} Confirmation of this suspicion will not serve as a useful diagnostic aid if the majority of MSI-H cancers detected in this way is sporadic and there is no reliable method of distinguishing the small subset occurring in a background of HNPCC.

Because the discrimination of MSI-H cancers has clinical implications for prognosis¹⁶ in sporadic disease and for risk assessment of the relatives of the HNPCC patient, this article examines the feasibility of distinguishing familial (HNPCC) and sporadic MSI-H CRC on the basis that these are not merely the bimodally distributed representatives of a single pathway of tumorigenesis.⁷ Apart from differences in age at presentation, up to 50% of HNPCC cancers are caused by a germline mutation in *hMSH2* whereas virtually all sporadic MSI-H cancers are associated with loss of expression of hMLH1.¹⁷ Occasional sporadic *hPMS2* mutations have been reported as the primary cause of colorectal cancers.¹⁸ Additionally, sporadic MSI-H cancers have been linked with the serrated pathway of tumorigenesis¹⁹ and a high frequency of mucinous differentiation with gastric mucinous metaplasia,²⁰ whereas HNPCC cancers are associated with traditional adenomas.²¹ The recognition of fundamental differences between sporadic and familial MSI-H CRC may not only translate into diagnostic algorithms but may also account for inconsistencies in the literature regarding the molecular genetic profile, natural history, and responsiveness to adjuvant chemotherapy of the MSI-H subset of CRC.

Materials and Methods

Samples

This study was performed on a selected group of 57 MSI-H sporadic colorectal cancers derived from patients with no evidence of family history of colorectal cancer and in whom germline mutations in *hMLH1* had been excluded by denaturing HPLC analysis. The patients that were chosen represented all of the MSI-H cases lacking family history from serial colectomies in two large public hospitals in Queensland, Australia. Age was not a criterion for ascertaining patients with sporadic MSI-H cancer. A further series of 112 MSI-H colorectal cancers from patients belonging to 73 families was studied. Of these, 49 fulfilled the original or modified Amsterdam criteria²² and the remaining 24 either satisfied the minimal Bethesda criteria,¹⁴ or were previously found to carry germline mutations in the mismatch repair genes *hMLH1*, *hMSH2*, or *hMSH6*. Patients provided informed consent in writing. Patient sex and age at onset of cancer were recorded. DNA was extracted from all fresh samples by a

Table 1. Details of Immunohistochemistry Antibodies

Antigen	Antibody clone	Dilution	Source
hMLH1	G168-15	1:75	BD PharMingen, San Diego, CA
hMSH2	G219-1129	1:150	BD PharMingen, San Diego, CA
hMSH6	GTBP.P1/2.D4	1:150	Serotec Laboratories Kidlington, UK
hPMS2	A16-4	1:75	BD PharMingen, San Diego, CA
β -catenin	Goat polyclonal	1:700	Santa Cruz Biotechnology Inc., Santa Cruz, CA
p53	D07	1:100	DAKOcorp, Carpinteria, CA

salt precipitation technique²³ and from all archival samples by a simple proteinase K digestion²⁴ followed by a phenol/chloroform purification step.

Microsatellite Instability Testing

MSI status was determined under the current recommendations of the National Cancer Institute Co-operative Family Registry for Colorectal Cancer Studies Biospecimens Subcommittee. Specimens were tested with a panel of 10 microsatellites (mononucleotides BAT-26, BAT-25, BAT-40, BAT-34C4, and higher order repeats MYCL, D10S197, D18S55, D5S346, D17S250, and ACTC) using techniques described in a previous report.²

Immunohistochemistry

Paraffin sections were affixed to Superfrost Plus adhesive slides (Menzel-Gläser; Braunschweig, Germany) and air-dried overnight at 37°C. After dewaxing and rehydration to dH₂O, sections for immunostaining for mismatch repair proteins were subject to heat antigen retrieval in 0.001 mol/L of ethylenediaminetetraacetic acid, pH 8.0, at 121°C in an autoclave on wet cycle. Those for staining for p53 and β -catenin were antigen retrieved in 0.01 of mol/L citric acid, pH 6.0. After cooling, the slides were thoroughly washed in Tris-buffered saline (TBS; 0.05 mol/L Tris, 0.15 mol/L NaCl), pH 7.2 to 7.4. Endogenous peroxidase activity was blocked using 1.0% H₂O₂, 0.1% NaN₃ in TBS. Nonspecific antibody binding was inhibited by incubating the sections in 4% skim-milk powder in TBS with the exception of slides for β -catenin staining. After transfer to a humidified chamber, the sections were incubated with 10% normal (nonimmune) goat serum (Zymed Corp., San Francisco, CA) or in the case of slides for β -catenin staining, nonimmune horse serum (Silenus Laboratories, Melbourne, Australia). Excess normal serum was decanted and the sections incubated overnight with primary antibody. The various primary antibodies are summarized in Table 1.

After washing in TBS, biotin-like activity was blocked using Biotin Blocking Kit (DAKO, Carpinteria, CA). The sections were subsequently incubated with biotinylated goat anti-mouse or donkey anti-goat immunoglobulins (Jackson ImmunoResearch, West Grove, PA) as appropriate at 1:300 dilution for 45 minutes then with streptavidin-horseradish peroxidase conjugate (Jackson ImmunoResearch) diluted 1:600 for 15 minutes. Color was developed in 3,3'-diaminobenzidine (Sigma Chemical, St. Louis, MO) with H₂O₂ as substrate. The sections were

washed in running tap water, lightly counterstained in Mayer's hematoxylin, dehydrated through ascending graded alcohols, cleared in xylene, and mounted using DePeX (BDH Gurr, Poole, UK).

Assessment of altered β -catenin expression was undertaken at the deep tumor margin and was based on: loss of lateral membrane expression (score 0 for present and 1 for absent), cytoplasmic staining (score 0, 1, or 2 for none, weak, and strong, respectively), and nuclear staining (score 0, 1, or 2 for none, weak, or strong, respectively). The final total of 0 to 5 was collapsed into β -catenin grade 1 (total 0 or 1), grade 2 (total 2 or 3), and grade 3 (total 4 or 5).²⁵ Sections stained for p53 protein were scored by two independent observers (MDW and JRJ). The proportion of positive cancer cell staining was graded as follows: 0, negative; 1, <10%; 2, 11 to 25%; 3, 26 to 50%; 4, 51 to 75%; and 5, and >75%. Only tumors scoring 3 or more were considered positive for p53 overexpression. Normal epithelium and stromal cells provided a positive internal control for mismatch repair proteins and β -catenin, whereas known p53-positive colorectal adenocarcinomas were stained with each batch.

Methylation Studies

A subset of tumors was screened for methylation in the promoter region of hMLH1 using methylation-specific PCR (MSP) and combined bisulfite restriction analysis (COBRA) assays.^{26,27}

Bisulfite Modification

Genomic DNA (300 ng) was diluted in 20 μ l of H₂O, denatured by treatment with 0.3 N NaOH, incubated at 75°C for 20 minutes, and quenched on ice. Freshly prepared hydroquinone (14 μ l of 10 mmol/L) and NaHSO₃ (250 μ l of 4.8 mol/L) were added to each denatured DNA sample. All samples were incubated under mineral oil at 55°C for 5 hours. The NaHSO₃-treated DNA samples were purified using Wizard DNA purification resin (Promega, Madison, WI), denatured with 0.3 N NaOH, precipitated with ethanol, and resuspended in 20 μ l of H₂O.

Methylation-Specific PCR (MSP)

MSP analysis was performed using primer pairs previously reported.²⁷ The polymerase chain reaction (PCR) mixture included: 10 $\times$ reaction buffer IV (AB gene), de-

oxynucleotide triphosphates (200 $\mu\text{mol/L}$), MgCl_2 (1.5 mmol/L), primers (16 pmol each per reaction), 2.5 μl of bisulfite-modified DNA, and 1.25 U of Red Hot DNA polymerase (*AB* gene) in a final volume of 40 μl . Amplification was performed in an MJ Research PTC-200 thermal cycler (MJ Research, Inc., Watertown, MA) for 35 cycles (1 minute at 95°C, 1 minute at 59°C, and 1 minute at 72°C) followed by a final 5-minute extension at 72°C. Fourteen μl of each PCR reaction product was loaded onto a 10% nondenaturing polyacrylamide gel and visualized by ethidium bromide staining.

Combined Bisulfite Restriction Analysis (COBRA)

Primary PCR reactions were performed in a volume of 25 μl containing 10 $\times$ reaction buffer IV (*AB* gene), dNTPs (250 $\mu\text{mol/L}$), MgCl_2 (1.5 mmol/L), primers (10 pmol each per reaction), 2 μl of bisulfite-modified DNA and 0.5 units of Red Hot DNA polymerase (*AB* gene). The amplification conditions consisted of a touchdown protocol with annealing temperatures decreasing from 59°C to 50°C every two cycles, followed by cycling at 50°C annealing temperature for 30 cycles. A nested PCR reaction was performed in a 50- μl volume using 1- to 2- μl PCR product from the primary reaction as template. The nested PCR products were digested with 8000 U of *RsaI* overnight at 37°C. Digested samples were analyzed on 15% nondenaturing polyacrylamide gels and visualized by ethidium bromide staining. The amount of hMLH1 methylation in each sample was determined using ImageQuant software and expressed as a percentage. *AluI* digests were performed to confirm that the sodium bisulfite conversion was complete. Cleavage with *AluI* will only occur if the recognition sequence (AGCT) has not been destroyed by bisulfite conversion. The primer sequences used for the primary COBRA assay were those reported elsewhere.²⁸ The sequences for the nested reaction were 5'-GATT-TAGTAATTTATAGAGT (sense) and 5'-AATACCT-TCAACCAATCAC (antisense). The primers for the MSP assay correspond to the CpG sites in the region -716 to -601 and the primers for the COBRA assay correspond to the CpG sites in the region -221 to -28.

Pathology Review

Data relating to cancer site and stage, specifically depth of invasion, nodal spread, and distant spread, were obtained from the pathological and clinical records of each patient. Sections from the primary tumor were reviewed by a single pathologist (JRJ) to assess additional pathological parameters according to published criteria and without knowledge of the status of the specimen (familial or nonfamilial origin). In specimens with two or more synchronous cancers, only the largest was assessed. The features included: 1) tumor margin classified as either expanding or infiltrating²⁹; 2) presence of poor differentiation whether or not this was the major component. Criteria for poor differentiation included poor gland development with epithelial cells being arranged in small

and irregular clusters, or as single cells as in signet ring cell carcinoma, or as solid sheets (medullary pattern) or in the form of trabeculae or islands³⁰; 3) mucinous carcinoma in which at least 50% of the tumor comprised lakes of mucin³⁰; 4) tumor-infiltrating lymphocytes based on the finding in a hematoxylin and eosin-stained section of at least four unequivocal intraepithelial lymphocytes in a single $\times 40$ field³¹; 5) peritumoral lymphocytes based on the finding of a cap or mantle of chronic inflammatory cells at the deepest point of direct spread³⁰; 6) Crohn's-like infiltrate scored on the basis of finding within a single $\times 4$ field of at least three nodular aggregates of lymphocytes deep to the advancing margin of the tumor³²; 7) presence of residual adenoma classified as tubular, tubulovillous, villous, or serrated³⁰; and 8) presence of tumor heterogeneity as defined by the finding of two or more distinct subclones distinguished on the basis of tumor type, grade of differentiation, or distinctive architectural property and described by others as a mixed or mosaic pattern.¹⁵

Loss of Heterozygosity Studies and Mutation Detection

A subset of tumors was examined for early traditional colorectal cancer mutations essentially as previously reported.²⁵ Loss of heterozygosity was analyzed on chromosome 5q using PCR loss of heterozygosity and restriction fragment length polymorphism (RFLP) markers within the APC gene to avoid the interference MSI would contribute to the reading of the assay. K-ras mutations in codons 12 were sought using mutation-specific RFLP analysis,³³ and tumors from both groups underwent mutation analysis in the coding repeats of TGF- β RII.³⁴

Statistical Analyses

Means and percentages of the various demographic, clinical, molecular, and pathological features of tumors were compared between HNPCC and sporadic MSI-H patient groups using *t*-tests and Pearson's chi-square. Where numbers for percentages were small, a Fisher's exact test was performed to compare groups. To evaluate the independent effects of the demographic, clinical, molecular, and pathological factors on patient group, a binary logistic regression was used. Those factors whose likelihood ratio statistic for inclusion into the model had a corresponding *P* value of >0.20 were removed from the final model.

Data were analyzed using the STATXACT package from Borland Inc. and SPSS for Windows Release 10.0 (SPSS Inc., Chicago, IL).

Results

Clinical Parameters

The mean age of cancer onset in patients with sporadic MSI-H tumors was significantly different, 74.5 years

Table 2. Molecular and Immunohistochemistry Features

Feature	HNPCC	Sporadic MSI-H	P value*
Average positive MSI markers	72% (17–100%)	87% (50–100%)	<0.001 [†]
MLH1-negative staining	51/105 (48%)	47/47 (100%)	<0.001
MSH2-negative staining	47/105 (45%)	0/47 (0%)	<0.001
β -catenin score (average)	1.9	0.8	<0.001 [†]
β -catenin score (>2)	29/86 (34%)	4/31 (13%)	0.02
p53 score (average)	1.3	2.0	<0.001 [†]
p53 score (>3)	6/75 (8%)	6/30 (20%)	0.08
K-ras codon 12 mutation	2/20 (10%)	0/23 (0%)	0.41
hMLH1 methylation	11/20 (55%)	20/23 (87%)	0.02

*Fisher's exact or Pearson's chi-squared test.

[†]t-test.

(range, 57 to 96 years) compared with 46.7 years (range, 18 to 80 years) for HNPCC patients ($P < 0.001$, t -test). Gender distribution also differed between the groups with females comprising 68% of sporadic MSI-H tumors and 50% of HNPCC ($P = 0.023$, chi-square test). Within HNPCC, the mean age of onset differed slightly between males and females but this was not significant (45.8 years for males and 48.0 years for females; $P = 0.45$ t -test).

Microsatellite Instability Testing

A summary of molecular and immunohistochemistry findings is given in Table 2. Examples of markers used in the study are shown in Figure 1. Sporadic MSI-H tumors were characterized by a very high level of MSI with a mean positive yield of 87% of markers (range, 50 to 100%) whereas HNPCC tumors scored significantly less (72%; range, 17 to 100%) ($P < 0.001$, t -test for independent samples). The mononucleotide marker BAT26 detected 57 of 57 (100%) sporadic tumors and 108 of 112 (96%) HNPCC tumors. Four HNPCC tumors, which did not show mutations in BAT26, did not express hMSH2 protein and showed levels of MSI ranging from 30 to 50% of markers.

Figure 1. Examples of microsatellite instability at D17S250, BAT26, D5S346, and MYCL in groups of normal mucosa/tumor (N/T) pairs. Asterisks indicate a positive result.

Immunohistochemistry Studies

Examples of immunohistochemistry staining for MMR proteins are given in Figure 2. All sporadic tumors tested showed loss of expression of hMLH1 (47 of 47, 100%). In contrast, loss of both major mismatch repair genes was seen in the HNPCC group, with 51 (48%) losing hMLH1 and 47 (45%) losing hMSH2. Of the cancers losing neither hMLH1 nor hMSH2, two lost hMSH6, five lost hPMS2, and one lost both hMSH6 and hPMS2. A further single tumor showed no loss of any of the four MMR proteins tested. In 47 tumors lacking hMLH1 from both groups, which were stained for hPMS2, there was concomitant loss of hPMS2 in all cases. Of 32 HNPCC tumors lacking hMSH2, 27 (84%) showed simultaneous loss of hMSH6. The average proportion of positive microsatellite markers did not vary between tumors deficient in hMLH1 and hMSH2. Tumors with immunohistochemistry indicating germline mutation of *hPMS2* or *hMSH6* were indistinguishable with respect to microsatellite status from those with results indicative of *hMLH1* or *hMSH2* mutations. The mean scores for β -catenin differed significantly between the two groups with HNPCC tumors showing higher levels of nuclear staining (1.9 versus 0.8, $P < 0.0001$, t -test). Low levels of nuclear p53 staining were seen in both groups.

Methylation Studies

Methylation of *hMLH1* was examined in a subset of tumors from both groups. Representative results are shown in Figure 3. The majority of sporadic tumors so tested (20 of 23, 87%) showed methylation of *hMLH1* by both MSP and COBRA. Three cases, all males, had tumors which were unmethylated at *hMLH1*. In the HNPCC group, 11 tumors of 30 tested were methylated at *hMLH1*. Of these 30 tumors, 20 were hMLH1-negative and all 11 tumors methylated at *hMLH1* fell into this subgroup (55% of hMLH1-negative tumors). Nine tumors of 11 methylated at *hMLH1* were proximal ($P = 0.02$, Fisher's exact test).

Loss of Heterozygosity Studies and Mutation Detection (Traditional Molecular Pathways)

Loss of heterozygosity at 5q was seen in 0 of 20 sporadic and 1 of 13 HNPCC tumors informative for at least one

Figure 2. Immunohistochemical demonstration of MMR gene deficiency. Brown color indicates the presence of the protein under study. **1** shows the concomitant loss of MLH1 and PMS2, and **2** shows the concomitant loss of MSH2 and MSH6 in the poorly differentiated cancer on the **right** of the respective figures. Note positive staining in stromal cells and normal mucosa in sections where MMR expression is lost.

polymorphic marker in the region of the APC gene. K-ras mutations in codon 12 were present in 0 of 23 sporadic and 2 of 20 HNPCC tumors examined. TGF- β RII mutations were found in similar proportions across both groups with 18 of 27 (66%) sporadic and 14 of 20 (70%) HNPCC tumors showing mutations.

Pathology Review

Table 3 shows pathology features analyzed between the two tumor groups, and the features are illustrated in Figure 4. Both tumor types commonly showed an expanding rather than infiltrative margin. Tumor-associated lymphocytes (Crohn's-like, peritumoral, and tumor-infiltrating) were also common but more frequently seen in HNPCC cancers. Although both groups were more likely to display proximal location, mucinous histology, and poor differentiation level than common colorectal cancer, these featured more often in the sporadic MSI-H tumors, reaching statistical significance in all three. The presence of subclones within the tumor (heterogeneity) was also more common in the sporadic group. A notable feature in subclones associated with sporadic MSI-H cancers was a serrated pattern reminiscent of serrated adenoma. Contiguous adenoma was observed in 30 cases (17 in HNPCC and 13 in sporadic MSI-H tumors). There was a striking difference in the histology of these contiguous lesions with 16 of 17 (94%) in HNPCC lesions comprising tubular or tubulovillous (traditional) adenomas contrasting with only 1 of 13 (8%) in sporadic cancers ($P < 0.001$). Conversely, 12 of 13 (92%) MSI-H sporadic cancers were adjacent to serrated adenomas. Adjacent serrated adenoma was observed in only 1 of 17 (6%) HNPCC cancers ($P < 0.001$).

Multivariate Analysis

A multivariate logistic regression was used to establish a predictive model for familial and sporadic tumors. Vari-

ables considered for inclusion in the model were age, sex, histological type of tumor (mucinous or nonmucinous), location of tumor in the colon (proximal or distal), the presence of tumor-infiltrating lymphocytes, of peritumoral lymphocytes, of subclones, of contiguous adenoma, and whether the tumor was poorly differentiated as well as the β -catenin score and the absence of hMSH2 staining. Those variables found to independently significantly predict sporadic tumors were increasing age ($P < 0.001$) and presence of subclones ($P = 0.025$). Presence of peritumoral lymphocytes significantly predicted HNPCC tumors ($P = 0.025$), and there was a tendency toward tumor-infiltrating lymphocyte presence predicting HNPCC tumors ($P = 0.149$). Based on the classification of these four features, the model was able to classify 94.5% of tumors as either sporadic or HNPCC.

Discussion

The patient of intermediate age (50 to 65 years) and uncertain family history presenting with an MSI-H colorectal cancer that is hMLH1-negative represents a clinical problem in that it is not immediately apparent whether the cancer has occurred sporadically or in the setting of an underlying germline mutation (HNPCC). In exploring this scenario, we have examined histopathology features, molecular changes, alterations in the immunohistochemical profile, methylation, and patient age and sex. Our findings suggest that familial and sporadic MSI-H cancers evolve through independent pathways although converge with respect to mismatch repair deficiency. During this work, contrasts emerged between these tumor types reflecting their origins in different precursor lesions.

We found the MSI-H sporadic cancer to be predominantly a late onset disorder of females and its familial counterpart to occur significantly earlier and to affect both sexes equally. HNPCC colorectal cancer has been

Figure 3. hMLH1 methylation studies on two normal mucosa/tumor (N/T) pairs. In both instances the normal mucosa/tumor pair on the **left** is from a methylated cancer and that on the **right** from an unmethylated cancer. **a:** The results of methylation-specific PCR. NU, normal mucosa DNA amplified with a methylation-insensitive primer; TM, cancer DNA amplified with a methylation-sensitive primer. Note that the unmethylated cancer is not detected using the methylation-sensitive primer. **b:** The COBRA assay. N, normal mucosa DNA; T, cancer DNA. Note that the PCR product has been cleaved into restriction fragments in the methylated cancer.

shown to be more common in males in a previous report, although females were more likely to develop sporadic MSI-H colorectal cancer³⁵ despite the reported association with cigarette smoking.³⁶ Females were also more likely to show methylation of *hMLH1*, a key mechanism in the genesis of sporadic MSI-H colorectal cancer.³⁵ Although familial and sporadic MSI-H cancers showed an overlapping age distribution, no sporadic cancers were seen in patients younger than 57 years. Hence patients presenting at 55 years and younger are highly likely to have HNPCC. Although HNPCC colorectal cancer occurred in young patients, sporadic MSI-H colorectal cancer was more age-related than either MSS or MSI-L colorectal cancer. The demographic data indicate a fundamental distinction of familial and sporadic MSI-H colorectal cancer.

MSI testing is used to define MSI-H colorectal cancer. In this study, all sporadic cancers were characterized by instability in at least 50% of markers, whereas HNPCC cancers showed significantly less extensive instability. All sporadic cancers lacked hMLH1 staining, whereas absent staining for a range of mismatch repair proteins was seen in HNPCC. All tumors lacking hMLH1 showed absence of hPMS2, whereas five lacked hPMS2 alone. The relation between hMLH1 and hPMS2 has been reported previously,^{18,37} and suggests that hPMS2 may be degraded in the absence of its binding partner, hMLH1, as hPMS2 mRNA is still abundant in tumors despite the lack of protein. Similarly, many tumors lacking hMSH2 had no staining with hMSH6, consistent with the findings of others.³⁸ Methylation seemed to be the predominant form of silencing of *hMLH1* in sporadic disease. However, it was not absent from HNPCC with more than half of hMLH1 tumors showing methylation presumably of the wild-type allele.³⁹ In HNPCC, this process was not restricted to females or to older patients, although it was more likely to be found in the proximal colon (data not shown).

Significant differences are apparent in the spectrum of molecular alterations in the two tumor sets. The most striking difference was seen in the *wnt* pathway where nuclear staining for β -catenin was a common feature in HNPCC. *K-ras* mutations in codon 12 were rare in both groups. Both groups displayed TGF- β RII mutations at rates of ~70%. Loss of 5q was not common in either group, despite the increased nuclear staining for β -catenin in familial tumors. It is likely that direct mutations in β -catenin itself or other genes in its signaling pathway such as *axin2* may be responsible for this observation in familial tumors.⁴⁰ Given the differences in the early morphogenesis of familial and sporadic MSI-H colorectal cancer, one would expect to observe matching differences in the molecular profiles of the two pathways. Studies including HNPCC cancers and/or MSI-H cell lines show evidence of disruption of the *wnt* signaling pathway as indicated by *APC* mutation,^{41,42} β -catenin mutation⁴³ or abnormal cytoplasmic and nuclear staining of β -catenin. Studies that focus on sporadic MSI-H colorectal cancer show little evidence of *APC* mutation,^{44,45} β -catenin mutation,⁴⁴ or abnormal immunolocalization of β -catenin.²⁵ Most studies show a trend toward or significant reduction of *K-ras* mutation in sporadic MSI-H cancers^{25,46} whereas codon 13 *K-ras* mutation occurs at a higher frequency in HNPCC.⁴⁷ These data are consistent with the differing routes of morphogenesis of familial *versus* sporadic MSI-H colorectal cancer, specifically the traditional adenoma-carcinoma sequence in the former and an alternative (serrated) route in the latter.

Although HNPCC has long been known to show a predilection for the proximal colon, up to 40% of cancers present in the distal bowel. Rectal cancers are a significant complication after total colectomy and ileorectal anastomosis.⁴⁸ By contrast, ~90% of sporadic MSI-H cancers occur in the proximal colon.⁴⁹ In using histological features to distinguish MSI-H cancers (regardless of whether these

Table 3. Clinical and Pathology Features

Feature	HNPCC	Sporadic MSI-H	P value*
Female sex	56/112 (50%)	39/57 (68%)	0.05
Age (average)	47 (18–80)	74.5 (57–96)	<0.001†
Expanding	77/81 (95%)	45/47 (96%)	1.000
Poor differentiation	28/81 (34%)	27/47 (57%)	0.02
Mucinous histology	16/72 (22%)	18/42 (43%)	0.02
Proximal location	62/91 (68%)	43/51 (84%)	0.04
Presence of sub-clones	14/53 (26%)	32/41 (78%)	<0.001
Crohn-like reaction	48/79 (61%)	22/45 (48%)	0.34
Tumour-infiltrating lymphocytes	58/79 (73%)	24/45 (56%)	0.08
Peritumoral lymphocytes	27/79 (34%)	8/45 (17%)	0.05
Contiguous adenoma	17/80 (21%)	13/42 (31%)	0.23
Contiguous serrated adenoma	1/80 (1%)	12/42 (29%)	<0.001
Contiguous traditional adenoma	16/80 (20%)	1/42 (2%)	<0.001

*Fisher's exact or Pearson's chi-squared test.
†t-test.

were sporadic or familial), Alexander and colleagues¹⁵ found the subjective interpretation of the overall histopathological appearance was more discriminating than any individual feature. No such attempt to apply an overall diagnosis of sporadic *versus* familial MSI-H cancer was used in this study. Nevertheless, experience derived in retrospect

allowed such a global impression to be formed. Overall, HNPCC cancers were more like traditional colorectal cancers apart from the higher frequency of lymphocytic infiltration. This was a feature of both familial and nonfamilial groups but peritumoral and tumor-infiltrating lymphocytes were more frequent in HNPCC cancers. Although the most

Figure 4. **A:** Poorly differentiated sporadic MSI-H adenocarcinoma showing epithelium arranged in irregular clusters and trabeculae. **B:** Medullary type and lymphocyte-rich carcinoma from subject with HNPCC. **C:** Poorly differentiated adenocarcinoma from subject with HNPCC carcinoma composed of epithelium arranged in islands. **D:** Well-differentiated mucinous carcinoma in which epithelium is lined by tall goblet cells and from a subject with HNPCC. **E:** Moderately differentiated adenocarcinoma from subject with HNPCC showing tumor-infiltrating (intraepithelial) lymphocytes. **F:** Residual serrated adenoma adjacent to a sporadic MSI-H colorectal cancer (not shown). **G:** Example of two distinct subclones, one poorly differentiated medullary type and the second mucinous from a sporadic MSI-H cancer. **H:** Sporadic MSI-H colorectal cancer formed of well-differentiated papillae covered by a serrated epithelium and associated with moderate amounts of extracellular mucin. **I:** Sporadic MSI-H colorectal cancer showing retention of serrated crypt morphology in association with abundant extracellular mucin. H&E; original magnifications: $\times 80$ (**A**, **F**, **G**); $\times 160$ (**B**, **C**, **D**); $\times 320$ (**H** and **I**); and $\times 800$ (**E**).

distinctive feature of HNPCC cancers was the pronounced lymphocytic infiltrate, sporadic MSI-H cancers showed several additional features distinguishing them from both HNPCC and common sporadic colorectal cancer. The frequency of poor differentiation, proximal location, and mucinous histology was higher in these cancers, and even nonmucinous carcinomas were likely to include fields containing pools of extracellular mucin (Figure 4, A-I).

Sporadic MSI-H cancers were more likely to show two or more subclones. These were distinguished on the basis of grade of differentiation, for example moderate and poor or type of cancer, for example mucinous and nonmucinous (Figure 4G). The mucinous areas in sporadic MSI-H cancers were more likely to be poorly differentiated and composed of ribbons, irregular cell clusters, or laciform structures. Mucinous change in HNPCC, by contrast, usually featured a well-differentiated columnar epithelium similar to the epithelium of villous adenoma. Areas showing obvious foci of serration occurred in many sporadic MSI-H cancers (Figure 4, H and I). In these fields and also in nonserrated areas, cytoplasm is typically abundant and eosinophilic whereas nuclei are vesicular and contain a prominent nucleolus. Residual serrated adenoma occurred in 12 sporadic MSI-H cases (Figure 4F), whereas residual traditional adenoma featured in 16 HNPCC cancers. In only one instance were remnants of traditional adenoma observed adjacent to a sporadic MSI-H and serrated adenoma adjacent to an HNPCC cancer. This finding adds further evidence to the case for a serrated precursor for these lesions. These findings are also supported by a recent report⁵⁰ that examined a series of 466 colorectal cancers for the presence of remnant serrated adenoma. These were found in 5.8% of cases and were significantly associated with the presence of MSI.

Previous reports that looked at the question of distinctive features in MSI-H cancers may have overlooked differences because of more permissive definitions of MSI-H cancers (which included some MSI-L cases) and because the cohorts studied pooled MSI-H sporadic cancers with those occurring in HNPCC. In this report, we show that MSI-H cancers occurring in HNPCC more closely resemble cancers developing by traditional pathways, both in morphology and mutation spectrum, whereas those in the sporadic setting show strong evidence of evolution from serrated precursors. Although this report does not separate the two types of cancer into nonoverlapping groups, and finds that no single assay can be used to unequivocally partition the two groups, the combination of age at diagnosis and three pathology features (tumor heterogeneity, peritumoral lymphocytes, and tumor-infiltrating lymphocytes) allowed 94.5% of MSI-H cancers to be classified as sporadic or HNPCC.

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