Mucin Core Protein Expression in Colorectal Cancers with High Levels of Microsatellite Instability Indicates a Novel Pathway of Morphogenesis

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INTRODUCTION

The morphological events underlying the evolution of colorectal cancer have been described as the adenoma-carcinoma sequence (1). The usual genetic accompaniment of the sequence has been referred to as the “suppressor” pathway in view of the loss of multiple tumor suppressor genes. The best studied of these is APC, implicated in adenoma initiation, and TP53, involved in the transition from adenoma to carcinoma (2, 3). The underlying mechanism is thought to be an acquired state of chromosomal instability (4).

A second genetic pathway is driven by a defect in DNA repair caused by the inactivation of DNA mismatch repair genes. This has been described as the “mutator” pathway because of the widespread accumulation of mutations in repetitive genomic sequences (2, 5, 6). Tumor suppressor genes are also targets in this pathway, but the repertoire is at least in part determined by the preferred selection of genes possessing short repetitive tracts within their coding sequences. In this regard TGFβRII (7) and BAX (8) might be viewed as substitutes for APC and TP53. DNA MSI is an important biomarker for the mutator pathway, which applies to about 10% of sporadic colorectal cancers (9–11) as well as to cancers complicating the autosomal dominant condition HNPCC (12).

The adenoma-carcinoma sequence is generally thought to apply in both the suppressor and mutator pathways. MSI-H in HNPCC adenomas is well documented (13–16) but is rarely reported in sporadic adenomas. One study found no MSI in sporadic adenomas (16), but the same authors subsequently reported MSI-L in 3 of 49 sporadic adenomas but no MSI-H (17). Others described MSI-L in 7 of 70 sporadic adenomas, but none was MSI-H (18). A single report documenting MSI in 20 of 73 proximal adenomas was not stringent either in excluding HNPCC or in distinguishing MSI-L and MSI-H (19).

It has been suggested that a subset of colorectal cancers may develop through a different route of morphogenesis. In 1990, Longacre and Fenoglio-Preiser (20) characterized a variant of conventional adenoma distinguished by a serrated architecture similar to the HP. They also showed that 37% of serrated adenomas contained foci of high-grade dysplasia, and that carcinoma-in-situ occurred in 10% of serrated adenomas. This pathway might, therefore, follow a relatively rapid course. In showing clonal relationships between the hyperplastic and dysplastic components of mixed polyps, the concept of a serrated pathway has been broadened to include HPs, mixed polyps, and...
serrated adenomas as a continuum (21). The same study demonstrated a high frequency of MSI in dysplastic epithelium within either mixed polyps or serrated adenomas; 14 (48%) of 29 showed MSI-L and 5 (17%) of 29 were MSI-H (21).

Mucins are a diverse family of high-molecular-weight glycoproteins that are widely expressed by epithelial tissues and characterized by the presence of tandem repeat sequences rich in serine and threonine residues that are highly O-glycosylated (22). Mucins can be classified as either membrane-associated or secretory glycoproteins. MUC1 and MUC4 encode transmembrane mucins that are expressed within the intestinal tract. MUC1 is highly expressed in carcinomas and has been implicated in colonic tumor progression and metastasis (23, 24). MUC1 has been shown to modulate cell adhesion (25, 26) and is involved in signal transduction (27). MUC4 contains two extracellular epidermal growth factor-like domains that may interact with members of the c-erb family of growth factor receptors and, thereby, modulate growth control (28, 29). The genes encoding MUC2, MUC5AC, MUC5B, and MUC6, all of which are gel-forming secretory mucins, are clustered on 11p15 (30). These mucins are produced and secreted by goblet cells and are the major constituents of mucus, which acts to lubricate and protect epithelial tissues (31).

To date, the expression of mucins has not been studied in colorectal cancers stratified by microsatellite status. The relevance of such a study is 2-fold: (a) with regard to the possible histogenesis of MSI-H cancer through a pathway of serrated neoplasia, we have previously shown (32) that serrated epithelial polyps are characterized by increased expression of intestinal mucin MUC2, inappropriate expression of gastric mucin MUC5AC, and reduced expression of MUC4; and (b) MSI-H cancers are distinguished from MSS/MSI-L cancers by a number of clinicopathological features. These include reduced aggressiveness and overrepresentation of mucinous cancer (33–36). It is possible that some of these features are related to differences in mucin expression. In particular, the overrepresentation of mucinous cancers among MSI-H colorectal cancers may be explained by an origin from serrated polyps that show up-regulation of secretory mucins MUC2 and MUC5AC (32).

MATERIALS AND METHODS

Patients. Colorectal cancer specimens were obtained, after informed consent, from 93 patients undergoing surgery at the Royal Brisbane Hospital between 1989 and 1997. Information about patient age, sex, tumor type, site, grade, and stage was obtained from the hospital charts. There were 45 male and 48 female subjects with an average age of 69 years (range, 27–88 years). All of the patients were interviewed to exclude a positive family history of colorectal cancer. Cancers were located in the proximal colon (40 tumors), distal colon (29 tumors), and rectum (23 tumors). All of the cancers showed at least submucosal invasion and ranged in size from 16–110 mm (mean size, 49 mm). Tumor type and grade of differentiation were classified histologically in accordance with WHO criteria (37). Of the 93 primary tumors, 74 were adenocarcinomas, and 19 were mucinous carcinomas. Tumors were staged according to the Union Internationale Contre Cancer (UICC) tumor-node-metastasis (TNM) classification (38). There were 14 stage I, 46 stage II, 20 stage III, and 12 stage IV carcinomas. An additional female subject, age 56 years, with hyperplastic polyposis and six synchronous colorectal cancers (two stage I, three stage II, one stage III) was included in this study. Among the 27 polyps obtained from her colectomy specimen and ranging in size from 5 to 12 mm were 5 HPs, 5 serrated adenomas, 13 mixed polyps, and 4 adenomas.

Monoclonal Antibodies. Monoclonal antibodies used were BC2 (1.5 μg·ml⁻¹) against MUC1 (39), 4F1 (4 μg·ml⁻¹) against MUC2 (40), M4.275 (5 μg·ml⁻¹) against MUC4 (41), and M1 (2 μg·ml⁻¹) against MUC5AC (Neomarkers, Fremont, CA; Ref. 42). BC2, 4F1, and M4.275 were generated using synthetic peptide sequences derived from the respective apomucin amino acid tandem repeat regions, and M1 was raised against human gastric mucin and subsequently was shown to recognize a COOH-terminal sequence of the MUC5AC core protein (43).

Immunohistochemistry. The specimens were fixed for 4–16 h in 10% neutral buffered formalin and then routinely were paraffin-embedded. Histologically normal mucosa from the margins of the specimens served as control tissue.

Serial sections were stained immunohistochemically as described previously (32). Paraffin sections (3–4 μm) were affixed to Superfrost Plus adhesive slides (Menzel-Gläser, Braunschweig, Germany) and were air-dried overnight at 37°C. Sections were dewaxed in xylol and rehydrated through descending graded alcohols to TBS [0.05 M Tris and 0.15 M NaCl (pH 7.2–7.4)]. Sections were incubated in 1% periodic acid against MUC5AC (Neomarkers, Fremont, CA) or in 0.1% porcine trypsin (ICN Biomedicals Australasia, Sydney, Australia) with 0.1% NaN₃ in TBS for 10 min to block endogenous peroxidase activity and then were washed in three changes of TBS for 5 min each. Nonspecific antibody-binding was inhibited by incubating the sections in 4% skim milk powder in TBS for 15 min, followed by a brief wash in TBS. The sections were then placed in a humidified chamber and incubated with 10% normal (non-immune) goat serum (Zymed Corp., San Francisco, CA) for 20 min. Excess normal serum was decanted from the sections, and the primary antibody was applied overnight at room temperature except for BC2 (MUC1), which was applied for 60 min.

Sections were washed in three changes of TBS for 5 min each—the first buffer change contained 0.5% v/v Triton X-100—and then were incubated with biotinylated goat-antimouse immunoglobulins (Zymed) for 30 min. Sections were washed again in three changes of TBS for 5 min each. Nonspecific antibody-binding was inhibited by incubating the sections in 4% skim milk powder in TBS for 15 min, followed by a brief wash in TBS. The sections were then placed in a humidified chamber and incubated with 10% normal (non-immune) goat serum (Zymed Corp., San Francisco, CA) for 20 min. Excess normal serum was decanted from the sections; and the primary antibody was applied overnight at room temperature except for BC2 (MUC1), which was applied for 60 min.

Sections were washed in three changes of TBS for 5 min each—the first buffer change contained 0.5% v/v Triton X-100—and then were incubated with biotinylated goat-antimouse immunoglobulins (Zymed) for 30 min. Sections were washed again in three changes of TBS for 5 min each (the first wash contained 0.1% v/v Triton X-100), were incubated with streptavidin-horseradish peroxidase conjugate (Zymed) for 15 min, and were washed in three changes of TBS for 5 min each. Color was developed in 3,3’-diaminobenzidine (Sigma Chemical Company, St. Louis, MO) with H₂O₂ as substrate for 5 min; then sections were washed in running tap water, lightly counterstained in Mayer’s hematoxylin, dehydrated through ascending graded alcohols, cleared in xylol, and mounted using DePeX (BDH Gurr, Poole, United Kingdom).

As negative controls, serial sections were stained as above.
but incubated either with TBS alone (i.e., with omission of the primary antibody) or with a nonspecific monoclonal antibody, 401.21, which is the same immunoglobulin isotype as BC2 and M4.275, and which is directed against α-gliadin from wheat gluten (45).

Sections were scored by two independent observers (A-E. B-H., J.R.J.) with conflicts resolved using a conference microscope. The proportion of positive cancer cell staining was graded as follows: (a) 0 (= negative); (b) <10% (1+); (c) 11–25% (2+); (d) 26–50% (3+); (e) 51–75% (4+); and (f) >75% (5+). The staining intensity of cancer cells was graded as weak (1+), moderate (2+), or strong (3+). The localization was classified as either cytoplasmic or cell membrane. Scoring results for intensity and proportion positive were combined by multiplication for each mucin core protein into a summary score. Summary scores were then classified as either high or low staining after consideration of the distribution within each summary score variable, to allow statistical comparison with other variables.

DNA Replication Error Assays. The specimens were derived from a set of sporadic cancers characterized previously for microsatellite status (35, 46). Six additional cancers that were obtained from the single subject with hyperplastic polyposis were also characterized. Briefly, DNA was extracted from fresh cancer tissue samples for all of the cases except for the tissue from the tumors from the subject with hyperplastic polyposis, which were formalin-fixed and paraffin-embedded. DNA was also extracted from matching germ-line control samples derived from either normal colonic mucosa or peripheral blood lymphocytes. The DNA samples were amplified using PCR at the following loci: MYCL, AT3, D2S123, F13B (47), BAT-26 and BAT-40 (48). The PCR reactions were performed in a final volume of 20 μl, containing 100 ng of genomic DNA, 20 pmol of each oligonucleotide primer, 1.25 μM dATP, 10 μM dCTP, dTTP, and dGTP, 6 μCi [α-32P]dATP, and 1 unit Taq polymerase (Boehringer Mannheim, Mannheim, Germany). Reaction conditions consisted of 3 min at 92°C, followed by 31 cycles (45 s at 94°C, 1.5 min at 55°C, 1.5 min at 72°C) followed by a final extension for 5 min at 72°C. The PCR products were electrophoresed on denaturing 5% polyacrylamide (19:1) gels and visualized by autoradiography. Cancers were classified as MSI-H if bandshifts occurred in at least 40% of loci (11).

Data Analysis. The χ2 test was used to test for significant associations between variables.

RESULTS

Immunohistochemical Localization of MUC1, MUC2, MUC4, MUC5AC. In the normal mucosa, MUC1 was expressed along the apical membrane and focally in the cytoplasm of goblet and columnar cells of the lower two-thirds of crypts. MUC1 immunoreactivity was often increased in cancers and seen within the glyocalyx intraluminal material and in intracytoplasmic lumina (invaginations of the apical cell membrane; Fig. 1A). The MUC2 precursor detected by the monoclonal antibody 4F1 was expressed focally in the perinuclear cytoplasm of normal goblet cells. In cancers, MUC2 was present in the cytoplasm of cells assumed to be of goblet cell lineage (Fig. 1B). In normal mucosa, MUC4 staining was seen in the cytoplasm of both goblet and columnar cells. Similar but reduced expression was observed within the cancers (Fig. 1C). In the normal cells, focal expression of MUC5AC was seen in the cytoplasm and mucous droplets of goblet cells in a minor proportion of crypts. MUC5AC staining was similar to that of MUC2 in cancers, although it was more often restricted to cells with an obvious goblet-cell theca (Fig. 1D).

Mucin Expression, Clinicopathological Features, and MSI Status. High expression of MUC1 was seen in 39 (53%) of 74 adenocarcinomas and in 12 (63%) of 19 mucinous cancers. MUC4 was highly expressed in 25 (34%) of 74 adenocarcinomas and in 9 (47%) of 19 mucinous cancer. There was no statistically significant correlation between MUC1 expression and age, sex, tumor stage, grade, site, type, and MSI-status (see Table 1). Similarly, there was no significant association between MUC4 expression and these features with the exception of tumor stage, in which there was high expression of MUC4 in 11 (79%) of 14 stage I tumors compared with only 22 (28%) of 78 higher-stage lesions (P = 0.003).

Forty-four (47%) and 38 (41%) of the 93 colorectal cancer samples showed positive staining for MUC2 and MUC5AC, respectively. High levels of MUC2 expression were not significantly associated with age, sex, tumor stage, or grade. However, there was a significant association between high MUC2 expression and localization in the proximal colon (P = 0.035), and strong associations with both mucinous tumor type (P < 0.0001) and MSI-H phenotype (P < 0.0001). Similar correlations were seen for MUC5AC, with no significant difference between MUC5AC expression and tumor stage and grade but with a trend toward higher expression of MUC5AC in the proximal colon (P = 0.056) and significant associations with mucinous cancer (P = 0.027) and MSI-H phenotype (P = 0.0003).

Combined MUC2+/MUC4+/MUC5AC+ Phenotype and Modified MUC2+/MUC4+/MUC5AC+ Phenotype. The mucinous phenotype with high expression of MUC2 and MUC5AC and low expression of MUC4 was seen in 5 (7%) of 74 adenocarcinomas and 8 (42%) of 19 mucinous cancers. There was no statistically significant correlation between age, sex, stage, or site and expression of this phenotype. There was a significant association between this mucinous phenotype and poorly differentiated cancer (P = 0.02), mucinous tumor type (P < 0.0001), and the MSI-H phenotype (P = 0.0024). Because loss of MUC4 was not associated with MSI-H status, associations with the modified mucinous phenotype MUC2+/MUC5AC+ were determined. This occurred in 15 (68%) of 22 MSI-H cancers, 3 (13%) of 24 MSI-L, and 7 (15%) of 47 MSS cancers (P = 0.0001). There were significant associations between the modified mucinous phenotype MUC2+/MUC5AC+ and grade (P = 0.02), type (P < 0.0001) and MSI-H status (P < 0.0001). There was also a trend (P = 0.053) between expression of the MUC2+/MUC5AC+ phenotype and tumor site. Of the MSI-H cancers with a mucinous phenotype, 10 of 10 showed high MUC2 staining compared with 9 of 12 MSI-H nonmucinous cancers; 9 of 10 showed high MUC5AC reactivity as opposed to 8 of 12 nonmucinous tumors; and 9 of 10 showed high MUC2 and MUC5AC reactivity as opposed to only 6 of 12 nonmucinous tumors.
Immunohistochemistry of mucin expression in a moderately differentiated mucinous cancer with MSI-H. A, MUC1 expression within the apical membrane and the abundant extracellular mucin; immunostaining with BC2. B, MUC2 expression within malignant cell cytoplasm; glycosylated MUC2 within the extracellular space is not immunostained; immunostaining with 4F1. C, weak and focal expression only of MUC4 in goblet and columnar cells; immunostaining with M4.275. D, MUC5AC expression in malignant cells; glycosylated MUC5AC within the extracellular space is not immunostained; immunostaining with M1. ×300.
Hyperplastic Polyposis. Among the six cancers from the subject with HPs, one was MSS, one was MSI-L, and four were MSI-H. Three of the MSI-H cancers showed the MUC2/MUC5AC phenotype. The fourth was positive for MUC5AC but lost MUC2 expression. Neither of the non-MSI-H cancers lost MUC4 or expressed MUC5AC, but both retained MUC2.

DISCUSSION

The definition of mucinous cancer requires that at least 50% of the cancer tissue be composed of mucus (37). About 10% of colorectal cancers are mucinous but a higher proportion is observed if the term mucinous is altered to imply up-regulation of secretory mucin regardless of the presence of abundant pools. If this more biologically relevant definition is adopted in the case of MUC2, then 44 (47%) of 93 of cancers in the present study were mucinous. This high figure is only partly explained by enrichment of the series with MSI-H cancers, of which 19 (86%) of 22 were mucinous according to the altered definition, because 25 (35%) of 71 MSS/MSI-L cancers were mucinous also. Similar findings were observed for the aberrantly up-regulated gastric mucin MUC5AC (Table 2). These observations indicate a switch toward the mucous cell lineage within MSI-H cancers.

The increase in MUC1 expression and decrease in MUC4 expression was independent of microsatellite status. Because neither MUC1 nor MUC4 were expressed differentially according to microsatellite status it is unlikely that either molecule would explain the reduced aggressiveness of MSI-H cancers (33–35). Indeed, MUC1 expression did not differ by stage, although loss of MUC4 was more evident in higher-stage tumors ($P < 0.003$).

Although the mucinous phenotype MUC2+/MUC4- /MUC5AC+ was associated with MSI-H cancers, the effect is explained by the changes in MUC2 and MUC5AC rather than MUC4. The association of the modified phenotype (MUC2+/MUC5AC+) with poor differentiation (Table 1) fits with the known tendency for MSI-H cancers to be poorly differentiated (33–36). Ten (45%) of 22 MSI-H cancers were poorly differentiated in this study, and 8 (80%) of 10 showed the MUC2+/MUC5AC+ phenotype. The modified phenotype MUC2+/MUC5AC+ was also highly correlated with typing as mucinous carcinoma. Twelve (63%) of 19 mucinous cancers showed this phenotype, which was seen in only 13 (18%) of 74 adenocar-

### Table 1

<table>
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<th>Variables</th>
<th>MUC1</th>
<th>MUC2</th>
<th>MUC4</th>
<th>MUC5AC</th>
<th>MUC2+/MUC5AC+</th>
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<td>8 (47%)</td>
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<td>&gt;60 (n = 76)</td>
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<td>38 (50%)</td>
<td>26 (34%)</td>
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<td>14 (31%)</td>
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<td>22 (46%)</td>
<td>20 (42%)</td>
<td>20 (42%)</td>
<td>15 (31%)</td>
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<tr>
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<td>I (n = 14)</td>
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<td>9 (64%)</td>
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<td>Rectum (n = 23)</td>
<td>12 (52%)</td>
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<tr>
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a NS, not significant; TNM, tumor-node-metastasis.
b Well and moderate versus poor.

### Table 2

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<th>High mucin expression</th>
<th>MSS (n = 47)</th>
<th>MSI-L (n = 24)</th>
<th>MSI-H (n = 22)</th>
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<tr>
<td>MUC1</td>
<td>23 (49%)</td>
<td>14 (58%)</td>
<td>14 (64%)</td>
<td>NS*</td>
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<tr>
<td>MUC2</td>
<td>15 (32%)</td>
<td>10 (42%)</td>
<td>19 (86%)</td>
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<td>6 (25%)</td>
<td>8 (36%)</td>
<td>NS</td>
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<td>MUC5AC</td>
<td>13 (28%)</td>
<td>8 (33%)</td>
<td>17 (77%)</td>
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<td>MUC2+/MUC4- /MUC5AC+</td>
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<td>2 (8%)</td>
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<td>MUC2+/MUC5AC+</td>
<td>7 (15%)</td>
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a NS, not significant.
cinomas (P < 0.0001). This fits with the overrepresentation of mucinous cancer among the MSI-H subset (33–36). There was a tendency for MUC2+, MUC5AC+, and MUC2+/MUC5AC+ cancers to be proximal, although this reached statistical significance only for MUC2 (Table 1). Again, this fits with the predilection by MSI-H cancers for the proximal colon. The mechanism for up-regulation of the mucinous phenotype in MSI-H cancers is unknown. One possibility could be abrogation of the TGF-β signaling pathway resulting in an uncontrolled autocrine effect. TGF-β is known to down-regulate colonicoyte differentiation, whereas goblet cells are resistant to the effects of TGF-β (49). Hakomori (50) described significant perturbations in O-glycosylation of tumor-associated mucins, predominantly in the form of carbohydrate chain truncation and aberrant sialation or fucosylation. The core protein epitopes that are recognized by many antimucin antibodies, including those used in this study, are potentially masked by glycosylation. It is conceivable that the increased immunoreactivity in tumors is attributable, at least in part, to the exposure of core protein that is available for antibody binding without necessarily having increased protein synthesis. However, there is no evidence to date that posttranslational modifications differ in colorectal cancers according to microsatellite status. It is more likely that the differences in the detection of core protein relate to actual mucin expression changes associated with secretory lineage differentiation (32).

In this study, the finding that the mucinous phenotype MUC2+/MUC5AC+ is seen in 15 (68%) of 22 MSI-H cancers but only 10 (14%) of 71 of the MSS/MS-L cancers (P < 0.0001) is unexpected insofar as it associates the mucinous phenotype of serrated adenomas with MSI-H colorectal cancer (32). Nevertheless, the data fit with our earlier observations in a series of sporadic serrated polyps in which 5 (17%) of 29 dysplastic samples from mixed hyperplastic/adenomatous polyps or serrated adenomas were MSI-H (21).

It could be argued that sporadic MSI-H cancers do arise from conventional adenomas as is the case in HNPCC adenomas (13–15, 51). This would be feasible if MSI-H were acquired at the point of transition from adenoma to carcinoma (analogous to TP53 in MSS cancers). However, this is not supported by molecular data. The tumor suppressor gene APC plays a critical role in the initiation of colorectal adenoma. MSI-H cancers would, therefore, be expected to show APC alterations if the adenoma-carcinoma sequence applied. Yet, an extremely low rate of APC mutation has been reported in MSI-H cancers (52, 53), even after full-length sequencing of APC derived from MSI-H colorectal cell lines (54). Two studies failed to show a single example of 5q loss of heterozygosity in a total of 29 MSI-H colorectal cancers (46, 52). Furthermore, aberrant (nuclear) immunolocalization of β-catenin is rarely observed in MSI-H cancers, which suggests that the entire WNT signaling pathway is intact (46). A higher frequency of APC mutation in MSI cancers (although still significantly lower then in MSS cancers) has been reported (55). However, 19 of the 52 cancers with DNA replication errors were from subjects with HNPCC and 6 of 9 DNA markers used in this study were for dinucleotide repeats, which may show instability in MSI-L cancers.

We suggested previously that MSI-L colorectal cancer might arise through neoplastic transformation of a HP (21). It now seems likely that some, if not all, MSI-H cancers arise through a similar morphogenetic pathway. This would explain the infrequency of both sporadic MSI-H adenomas and APC alterations in MSI-H cancers. The mechanism could be through transformation of a HP or a serrated adenoma arising de novo. We do not believe that all of the colorectal cancers arising through the serrated pathway would be MSI-H. Although we have observed MSI-H cancers arising in association with hyperplastic polyposis (56), MSS and MSI-L cancers occur in the same condition (57). Similar findings were seen in the subject with multiple colorectal cancers and hyperplastic polyposis described in this study. However, it seems probable that many, if not all, MSI-H cancers do arise through the alternative serrated pathway. Given the numerous clinical, pathological, and molecular differences between MSI-positive and MSI-negative cancers that have now been demonstrated, it would perhaps be surprising if differences in early morphogenesis did not exist also.

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