DNA microsatellite instability and mismatch repair protein loss in adenomas presenting in hereditary non-polyposis colorectal cancer

H Iino, L Simms, J Young, J Arnold, I M Winship, S I Webb, K L Furlong, B Leggett, J R Jass

Abstract

Background and aim—Hereditary non-polyposis colorectal cancer (HNPCC), as its name implies, is associated with few adenomas, and the early evolution of colorectal neoplasia is poorly understood. In this study our aim was to clarify the genetic profiles of benign polyps in subjects with HNPCC using a combined molecular and immunohistochemical approach.

Methods—Thirty adenomas and 17 hyperplastic polyps were obtained from 24 affected HNPCC subjects. DNA was extracted from paraffin embedded tissue by microdissection and analysed for the presence of microsatellite instability (MSI) and mutations in five genes known to be targets in mismatch repair deficiency (TGF\betaR1, IGF2R, BAX, hMSH3, and hMSH6). Serial sections were stained by immunohistochemistry for hMLH1 and hMSH2.

Results—Twenty four (80%) of 30 adenomas showed MSI. Of MSI positive adenomas, 66.7% showed MSI at more than 40% of markers (high level of MSI (MSI-H)). Two of 17 hyperplastic polyps revealed MSI at one marker (low level of MSI (MSI-L)). A significant association was found between MSI-H and high grade dysplasia in adenomas (p=0.004). Eight of nine adenomas with mutations of coding sequences revealed high grade dysplasia and all nine were MSI-H. Four of the nine ranged in size from 2 to 5 mm. The presence of the hMSH6 mutation was significantly correlated with high levels of MSI (80% of markers) (p<0.02). Twenty four adenomas gave evaluable results with immunohistochemistry. One of six (17%) microsatellite stable, six of seven (86%) MSI-L, and 11 of 11 (100%) MSI-H adenomas showed loss of either hMLH1 or hMSH2.

Conclusions—Most adenomas in subjects with a definite diagnosis of HNPCC show MSI (80%). The finding of MSI-L is usually associated with loss of expression of hMLH1 or hMSH2, unlike the situation in MSI-L sporadic colorectal cancer. The transition from MSI-L to MSI-H correlated with the finding of high grade dysplasia and mutation of coding sequences and may be driven by mutation of secondary mutators such as hMSH3 and hMSH6. Advanced genetic changes may be present in adenomas of minute size.

Keywords: adenoma; microsatellite instability; mismatch repair; hereditary non-polyposis colorectal cancer

The early evolution of colorectal neoplasia in hereditary non-polyposis colorectal cancer (HNPCC) is poorly understood. While it is sometimes possible to demonstrate residual adenomas adjacent to HNPCC cancers, the use of somatic microsatellite mutations as molecular clocks discloses surprisingly long periods of parallel evolution of adenomas and carcinomas. This suggests that adenomas and carcinomas may not be so much sequentially related but derived from a common precursor lesion of microscopic dimensions. This could explain in part the well authenticated observation of interval cancer in HNPCC in which a patient presents with a cancer soon after a negative colonoscopic examination. Nevertheless, while adenomas are uncommon in HNPCC subjects, a disproportionate number are diagnosed before the age of 50 years, and they are more likely to be large, have a villous morphology, and show high grade dysplasia. These observations have introduced the concept of “aggressive adenoma” in HNPCC in which rapid evolution is driven by the acquisition of genetic instability due in turn to breakdown of the DNA mismatch repair mechanism.

DNA mismatch repair deficiency leads to a high level of microsatellite instability (MSI-H) in cancer tissue. It is generally agreed that p53 mutation and 17p and 18q loss of heterozygosity are infrequent in MSI-H cancers, whether HNPCC or sporadic. Genes with repetitive tracts in their coding sequences appear to provide an alternative molecular pathway in the evolution of MSI-H colorectal cancers. These include TGF\betaR1, IGF2R, BAX, CDX-2, E2F-4, and caspase 5. However, while microsatellite instability (MSI) may be established at an early stage in adenoma formation, including microscopic aberrant crypt foci, mutated genes have not been

Abbreviations used in this paper: HNPCC, hereditary non-polyposis colorectal cancer; MSI, microsatellite instability; MSS, microsatellite stable; PCR, polymerase chain reaction; TBS, Tris buffered saline.
detected at high frequency in adenomas showing MSI.14 15 Mutation of TGFβRII has been described as a very early and possibly initiating event16 whereas others link the TGFβRII mutation to the stage of adenoma-carcinoma transition.18 BAX mutations have also been related to adenoma-carcinoma transition.19 The extent of genetic instability appears to correlate with the risk of adenoma progression.20 It has been suggested that the full development of the microsatellite mutator phenotype depends on the recruitment of “secondary” mutators.21 Among these are hMSH3 and hMSH6, mismatch repair genes that are susceptible to the development of frameshift somatic mutations by virtue of their repetitive encoding sequences.21

The aim of this study was to stratify a series of adenomas obtained from subjects with a definite diagnosis of HNPPC according to microsatellite status (MSS), MSI-low (MSI-L) and MSI-H and to assess critically the timing of loss of expression of hMLH1 and hMSH2 and recruitment of secondary mutators (MSH3 and MSH6). By correlating these observations with demonstration of mutations in TGFβRII, IGFR2, and BAX as well as the classical morphological risk factors for adenoma progression, it should be possible to derive a clearer picture of the molecular steps governing the origin of cancer in HNPPC.

Materials and methods

Seventeen hyperplastic polyps and 30 adenomas were obtained from 24 affected HNPPC subjects as paraffin embedded archival samples. All families met stringent criteria for HNPPC as defined by the International Collaborative Group for HNPPC.22 Additionally, at least two cancers per family were required to show high levels of DNA MSI. Affected subjects were recruited either on the basis of developing an MSI-H colorectal cancer or a HNPPC associated cancer at less than 50 years of age. Twenty two (73.3%) adenomas were derived from 10 subjects (members of four different families) carrying a germline mutation of hMSH2. Despite the small size of the adenoma, all nine microsatellite loci showed instability, as did hMSH6 (not shown). N, normal; T, tumour DNA.

DNA PREPARATION AND ANALYSIS OF MICROSATELLITE INSTABILITY

DNA was extracted from microdissected sections of paraffin embedded archival sample as described previously.23 DNA from each pair of samples, normal mucosa and tumour, was analysed for MSI at nine loci: MYCL,24 D2S123,25 D5S346,26 D10S197,27 D18S58,28 BAT25,29 BAT26,30 BAT40,31 and c-mycT22. The loci D10S197 and D18S58 were analysed only in the case of polyps classified as MSS, MSI-L, or borderline MSI-H with seven loci. The reaction volume for the polymerase chain reaction (PCR) was 10 μl which included 2 μl of the DNA sample, 10 nM of each primer, 2 nM of each deoxynucleotide triphosphate, 1 μl of PCR buffer, 2.5 U of Taq polymerase (Boehringer Mannheim, Mannheim, Germany), and 1 μl of [33P]dATP. PCR was run as previously described.32 After amplification, PCR products were separated by electrophoresis in 5% denaturing polyacrylamide (19:1) gel and visualised by autoradiography. Tumours showing bandshifts at one or more microsatellite markers were termed MSI (fig 1). MSI status was subclassified as high (MSI-H) when bandshifts were seen at three markers (including at least one mononucleotide marker) and low (MSI-L) when less instability was detected. Scoring of MSI was undertaken independently by two observers (HI, JY).

Table 1  Pathological features of hereditary non-polyposis colorectal cancer polyps

<table>
<thead>
<tr>
<th>Location</th>
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<th>Adenomas</th>
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<td>8</td>
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<td>Distal colon</td>
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<tr>
<td>Dysplasia</td>
<td>Low grade</td>
<td>13</td>
</tr>
<tr>
<td>High grade</td>
<td>—</td>
<td>17</td>
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<td>17</td>
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Figure 1  Sample of four loci showing bandshifts, including three microsatellite loci (D2S123, BAT40, and c-myc) and the poly(A) tract of hMSH3. DNA was extracted from a 2 mm tubular adenoma from a patient carrying a germline mutation of hMSH2. Despite the small size of the adenoma, all nine microsatellite loci showed instability, as did hMSH6 (not shown). N, normal; T, tumour DNA.

Figure 2  Frequency of bandshifts in microsatellite markers used to distinguish MSI-H and MSI-L adenomas. All markers are sensitive for MSI-H adenomas whereas dinucleotide markers (particularly DSSS46, D10S197, and D18S58) are sensitive for MSI-L adenomas.
MUTATION ANALYSIS OF REPEAT SEQUENCES OF TGFβRII, IGF2R, BAX, hMSH3, AND hMSH6 GENES

The presence of mutation of the poly(A)10 tract of TGFβRII, the poly(G)8 tract of IGF2R, the poly(G)8 tract of BAX, the poly(C)8 tract of the hMSH3 gene, and the poly(C)8 tract of the hMSH6 gene was investigated by PCR and subsequent polyacrylamide gel electrophoresis. PCR was undertaken as for MSI testing. After amplification, PCR products were separated by electrophoresis in 5% or 8% denaturing polyacrylamide (19:1) gel according to the size of products, and visualized by autoradiography. The presence of bandshifts or an additional band was interpreted as a mutation (fig 1).

IMMUNOHISTOCHEMISTRY

Paraffin sections (4 µm) were fixed to Superfrost Plus charged slides (Menzelgläser, Braunschweig, Germany) and dried overnight at 37°C. The sections were dewaxed and rehydrated to distilled water through descending graded alcohols. Heat antigen retrieval was performed by autoclaving the sections in 0.001 M EDTA (pH 8.0) for 15 minutes. After cooling, the sections were transferred to Tris buffered saline (TBS), pH 7.4. Endogenous peroxidase activity was quenched by incubating in 1% H2O2 and 0.1% NaN3 in TBS for 10 minutes. Following thorough washing in TBS, sections were immersed in 4% commercial non-fat skim milk powder in TBS for 15 minutes to inhibit non-specific antibody binding, before being transferred to a humidified chamber and covered with 10% normal (non-immune) goat serum for 30 minutes. Excess serum was decanted from the sections which were then incubated overnight at room temperature with primary monoclonal antibodies to hMLH1 and hMSH2. The monoclonal antibodies G168-15 (anti-hMLH1 diluted 1:75 in TBS) and Ab-2 (anti-hMSH2 diluted 1:120) were obtained from PharMingen (San Diego, California, USA) and Oncogene Research Products (Cambridge, Massachusetts, USA), respectively.

Following the incubations, sections were washed thoroughly in three changes of TBS for five minutes each. Sections were then incubated for 45 minutes with appropriate biotinylated secondary antibodies (Zymed Laboratories, San Francisco, California, USA), and then streptavidin-horseradish peroxidase conjugate (Zymed) for 15 minutes. Antigenic sites were revealed by incubating sections in 0.05% 3,3'-diaminobenzidine in Tris saline with H2O2 as substrate. After washing in gently running tap water, the sections were lightly counterstained with haematoxylin, dehydrated through graded alcohols, cleared in xylene, and mounted with DePeX.

STATISTICAL ANALYSIS

Associations between clinicopathological variables and the frequency of genetic changes were assessed by χ2, Fisher’s exact test, or the Wilcoxon rank sum statistic using a Statxact package. Probability (p) values of <0.05 were considered significant.

Results

PATHOLOGICAL FEATURES OF POLYPS FROM HNPCC SUBJECTS

The sites and pathological features of the 47 colorectal polyps are summarised in table 1. Seven of 14 adenomas (50%) measuring less than 5 mm, six of nine adenomas (66.7%) measuring 5–9 mm, and four of seven adenomas (57.1%) of ≥10 mm revealed high grade dysplasia. There was no correlation between adenoma size and grade of dysplasia (Wilcoxon rank sum statistic, p=0.619).

FREQUENCY OF MICROSATELLITE INSTABILITY

Informative PCR products were obtained from 60% of the nine microsatellite markers and bandshifts at four loci are shown for an MSI-H adenoma (fig 1). Bandshifts occurred with similar frequencies in MSI-H adenomas regardless of marker type whereas dinucleotide markers were more sensitive for MSI-L adenomas (fig 2). Two of 17 (11.8%) hyperplastic polyps showed MSI at one marker (DSS346). No hyperplastic polyp was classified as MSI-H. Twenty four (80%) of 30 adenomas revealed MSI. Eight of 24 (33.3%) adenomas were classified as MSI-L and 16 of
24 (66.7%) as MSI-H. Only one MSI-L adenoma showed bandshifts at more than one locus (three dinucleotide loci). Bandshifts were seen at 10/63 (16%) evaluable loci in MSI-L adenomas. In contrast, 67/93 (72%) evaluable loci in MSI-H adenomas showed bandshifts. Twelve mutations in coding regions were identified in nine adenomas from six HNPCC subjects. No coding region mutations were identified in hyperplastic polyps (table 2).

**MSI STATUS AND CLINICOPATHOLOGICAL FEATURES OF HNPCC POLYPS**

Adenomas displaying high grade dysplasia revealed MSI-H more frequently than those with low grade dysplasia (Fisher’s exact test, p=0.004). There was no significant association between MSI status and sex, age, location, or size of adenomas. Nine of 14 (64.3%) adenomas less than 5 mm in size, four of nine (44.4%) of 5-9 mm, and three of seven (42.9%) more than 9 mm showed MSI-H (table 3). The two hyperplastic polyps classified as MSI-L were obtained from the proximal colon. One was 5 mm and another 10 mm in size.

**MUTATION OF REPEAT SEQUENCES IN CODING REGIONS OF TGF\(\alpha\)RII, IGF2R, BAX, hMSH3, AND hMSH6**

Mutations in repeat sequences of coding regions were detected in nine of 30 adenomas. One mutation was identified at TGF\(\alpha\)RII, two mutations at IGF2R and BAX, three mutations at hMSH3, and four mutations at hMSH6. Two adenomas showed mutations at two genes, one at IGF2R and hMSH6 and the second at hMSH3 and hMSH6. All nine adenomas with mutations in coding regions were MSI-H and eight (88.9%) showed high grade dysplasia. Three were only 2 or 3 mm in size and six of nine (66.7%) were less than 1 cm.

**IMMUNOHISTOCHEMISTRY**

Twenty four adenomas gave evaluable results with immunohistochemistry (fig 3). Distribution according to microsatellite status and expression of hMLH1 and hMSH2 are shown in table 5. One of six MSS adenomas showed MSI at 40–59% of markers, three at 60–79%, and seven at 80% or more. Four of four cases which showed mutations at hMSH6 revealed MSI at more than 80% of microsatellite markers (table 4). There was a significant association between MSI involving 80% of loci or more and mutation of hMSH6 (p<0.02).

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**Table 4** Relationship between microsatellite instability status and mutations in coding region of hereditary non-polyposis colorectal cancer adenomas

<table>
<thead>
<tr>
<th>%MSI</th>
<th>n</th>
<th>TGF(\alpha)RII(A)</th>
<th>IGF2R(G)</th>
<th>BAX(G)</th>
<th>hMSH3(A)</th>
<th>hMSH6(C)</th>
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<td>&lt;40</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>40–59</td>
<td>6</td>
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<td>1</td>
<td>1</td>
<td>1</td>
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<tr>
<td>60–79</td>
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<tr>
<td>80+</td>
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<td>1</td>
<td>1</td>
<td>1</td>
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<td>1</td>
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</tbody>
</table>

*Percentage of non-coding region markers with genetic alteration out of the informative markers; \(TGF\alpha\)RII(A)10, poly(A)10 tract of TGF\(\alpha\) receptor type II gene; \(IGF2R\)(G)8, poly(G)8 tract of IGF receptor type 2 gene; \(BAX\)(G)8, poly(G)8 tract of BAX gene; \(hMSH3\)(A)8, poly(A)8 tract of human MSH3 gene; \(hMSH6\)(C)8, poly(C)8 of human MSH6 gene.

**Table 5** DNA mismatch repair (MMR) protein expression in hereditary non-polyposis colorectal cancer adenomas, familial colorectal cancers (CRC), and sporadic colorectal cancers

<table>
<thead>
<tr>
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<th>HNPCC adenomas</th>
<th>Familial CRC†</th>
<th>Sporadic CRC</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MSS/MSI-L (n=13)</td>
<td>MSI-H (n=11)</td>
<td>MSS (n=21)††</td>
</tr>
<tr>
<td>No loss</td>
<td>6</td>
<td>0</td>
<td>20 (2)</td>
</tr>
<tr>
<td>hMLH1 loss</td>
<td>2</td>
<td>2</td>
<td>0 (13)</td>
</tr>
<tr>
<td>hMSH2 loss</td>
<td>5</td>
<td>9</td>
<td>9 (10)</td>
</tr>
</tbody>
</table>

Loss of MMR protein in MSS/MSI-L lesions: (a) HNPCC adenoma v familial CRC (p=0.001, Fisher’s exact test); (b) HNPCC adenoma v sporadic CRC (p<0.001, Fisher’s exact test).

†At least two first degree relatives with CRC; ††majority will not be HNPCC; †††majority will be HNPCC.

*Borderline MSI-L/MSI-H; **aged 34 and adopted (likely HNPCC).
Adenomas in HNPCC

**Discussion**

In this study, 80% of colorectal adenomas from subjects with HNPCC displayed DNA microsatellite instability (MSI). Others have shown that 57–93% of HNPCC adenomas reveal MSI. Using 26 dinucleotide microsatellite loci, Jacoby and colleagues found that a lower proportion of mutated microsatellite loci were detected in completely benign adenomas than in the benign areas of adenomas with foci of malignancy. The stage of MSI-L appears to be a distinct and perhaps early phase, associated primarily with dinucleotide instability (fig 2). Whether MSI-H adenomas must pass through an MSI-L stage or whether the MSI-H phenotype can arise de novo is unknown. It is of interest that one of six MSS adenomas showed loss of hMLH1 protein and six of seven MSI-L adenomas showed loss of hMLH1 (one) or hMSH2 (five) protein (table 5). The MSS adenoma with loss of hMLH1 was from a subject with a germline mutation of hMLH1. No bandshifts were seen at six of nine loci with evaluable PCR products, including BAT25 and BAT40. These findings are in contrast with sporadic and familial MSS/MSI-L colorectal cancers (table 5). This would suggest that MSI-L may occur as a stage in the evolution of MSI-H and that a percentage of MSI-L and MSI-H appear to be emerging phenotypes in sporadic colorectal cancer. The rarity of MSI-L (and MSS) colorectal cancer in HNPCC (we found two MSI-L and 68 MSI-H cancers in subjects with HNPCC—unpublished observations) suggests that the transition from MSI-L to MSI-H is occurring at the stage of adenoma in HNPCC. Cawkwell et al found a similar association between MSI-H status and loss of DNA mismatch repair gene expression in colorectal cancer. It is likely that MSI-H adenomas are more prone to progress to cancer. This would be consistent with our finding of a significant association between MSI-H status and high grade dysplasia (p=0.004).

It is noteworthy that 11 of 14 (78.6%) adenomas less than 5 mm in size showed MSI and nine of these (81.9%) were MSI-H. The smallest adenoma with MSI-H in this study was 2 mm in size and more than 50% of adenomas less than 5 mm in size revealed high grade dysplasia. MSI has been described in foci of colorectal neoplasia of microscopic size (aberrant crypt foci). Multiple aberrant crypt foci developed in the transgenic mucosa of MSH2-deficient mice. It is evident that the mutator phenotype (MSI-H as well as MSI-L) is established at an early stage of adenoma formation in HNPCC subjects. This may explain the high frequency of interval cancer.

TGFβRII and IGF2R participate in the control of epithelial cell growth whereas BAX regulates apoptosis. These genes have repetitive tracts in their coding sequences and are frequently mutated in the colorectal cancers of HNPCC or sporadic MSI-H cancers. hMSH3 and hMSH6 participate in the DNA repair system by forming complexes with the MSH2 protein. hMSH3 and hMSH6 have repeat sequences in their coding regions and frameshift mutations have been detected in HNPCC cancers and sporadic cancers showing MSI. Germline mutations of hMSH6 have also been reported. Mutation of TGFβRII or BAX was found in 63–90% of sporadic MSI-H cancers and in 54% of HNPCC cancers. In adenomas, the frequency of mutation of these genes was 50–64% and 15%, respectively. These findings relate mutation of TGFβRII with early adenoma progression and BAX with the transition from adenoma to carcinoma.

In the present study, nine of 16 (56%) MSI-H adenomas were found to have mutations at one or more coding regions while none of 14 MSI-L or MSS cases showed coding region mutations. This fits with the low frequency of mononucleotide instability in MSI-L lesions (fig 2) as target coding sequences comprise mononucleotide runs. There was one (6.3%) mutation of TGFβRII and two mutations of IGF2R (12.3%) and BAX (12.6%). Three (18.8%) and four (25.0%) of 16 MSI-H adenomas had mutations at hMSH3 and hMSH6, respectively. No adenoma had mutations at more than two coding regions. Compared with earlier studies the frequency of mutation in coding regions, particularly TGFβRII, was low. These data suggest that mutations of TGFβRII, IGF2R, and BAX follow no sequential pattern but cluster within adenomas showing MSI-H and high grade dysplasia (table 4). Other genes, including CDX-2, E2F-4, caspase-5, and as yet unknown genes may also be involved in adenoma progression.

Although HNPCC patients develop adenomas at about the same rate as the general population, HNPCC adenomas are more prone to malignant conversion than their sporadic counterparts. Somatic mutations of additional mismatch repair genes may serve as secondary mutators and accelerate the accumulation of MSI and further coding region mutations. Our data suggest that hMSH6 and hMSH3 may function as secondary mutators. The hMSH6 mutation occurred only in adenomas with the highest levels of MSI (table 4). However, the possibility that mutation of MSH6 could be an epiphenomenon, secondary to the establishment of high levels of MSI-H, cannot be excluded. Furthermore, our data suggest that mutation of hMSH3 and hMSH6 cannot be the only explanation for the critical transition from MSI-L to MSI-H.

In spite of the right sided predominance of colon cancer in HNPCC and MSI positive sporadic colon cancers, our data showed no significant association between location of adenoma and MSI (table 3). Of nine adenomas with mutation of coding genes, only two were derived from the proximal colon. Jacoby and colleagues and Akiyama and colleagues also indicated no site predilection for HNPCC adenomas. The two hyperplastic polyps with MSI were obtained from the proximal colon. One hyperplastic polyp with MSI and TGFβRII mutation has been reported. The current data provide little evidence that sporadic hyperplastic polyps play a role in the
evolution of colorectal cancer in HNPCC but do not exclude a minor role in the case of proximally located polyps. The six MSS adenomas could be coincidental sporadic adenomas, a suggestion that would be supported by an age relationship and retention of expression of hMLH1 and hMSH2 in five of the six adenomas. In fact, three MSS adenomas occurred in subjects aged less than 40 (table 3). It appears that even colorectal cancers may occasionally be MSS in individuals carrying a mismatch repair gene germline mutation. In conclusion, most colorectal adenomas in HNPCC show low or high levels of DNA microsatellite instability and most of these show loss of hMLH1 or hMSH2. The transition to high grade dysplasia is associated with the MSI-H phenotype and is accompanied by mutation of coding sequences in TGFBR2, IGF2R, and BAX. MSH3 and MSH6 may add to the MSI burden as secondary mutators.

We thank Beenda Mason for secretarial support and Clay Waterford for photographic assistance. Supported by NIH/USAM grant No 1 U01 CA74778–01 (Collaborative Family Registry for Colorectal Cancer Studies).