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# Microsatellite Instability and Expression of MLH1 and MSH2 in Carcinomas of the Small Intestine

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**BACKGROUND.** Carcinomas of the small intestine are rare, but the risk is greatly increased in patients with hereditary nonpolyposis colorectal cancer (HNPCC) due to an inherited mismatch repair (MMR) gene mutation, most commonly affecting the genes *MLH1* or *MSH2*. Defective MMR is characterized by microsatellite instability (MSI) and loss of MMR protein expression in the tumor tissue. However, a subset of several sporadic tumor types, including about 15% of colon cancers, also evolve through defective MMR.

**METHODS.** The authors have assessed the frequency of MSI and analyzed the immunohistochemical expression of MLH1 and MSH2 in a population-based series of 89 adenocarcinomas of the small intestine. To study the contribution of MSI and defective MMR protein expression in young patients, 43 cancers of the small intestine from patients below age 60 years (including 24 tumors from the population-based series and an additional 19 tumors from young individuals) were also analyzed.

**RESULTS.** MSI was detected in 16/89 tumors (18%) in the population-based series, and immunohistochemistry revealed loss of expression for MLH1 in 7/16 MSI tumors and in 2/73 MSS tumors, whereas all tumors showed normal expression for MSH2. Among the young patients, the authors identified MSI in 10/43 tumors (23%), and 6 of these 10 MSI tumors showed immunohistochemical loss of MMR protein expression, which affected MLH1 in 3 cases and MSH2 in 3 cases.

**CONCLUSIONS.** The frequency of MSI (18%) in adenocarcinomas of the small intestine equals that of colon cancer. However, silencing of MLH1 seems to explain the MSI status in only about half of the MSI tumors. Among patients with cancer of the small intestine before age 60 years, MSI is found in 23% of the cases, with MLH1 and MSH2 being affected at equal frequencies, indicating that HNPCC may underlie a subset of such cases. *Cancer* 2003;97:1551-7.

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**KEYWORDS:** adenocarcinoma, small intestine, mismatch repair, microsatellite instability, hereditary nonpolyposis colorectal carcinoma.

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**M**alignant tumors of the small intestine account for less than 5% of all gastrointestinal malignancies, although the small intestine constitutes about 75% of the length of the gastrointestinal tract.<sup>1</sup> In Sweden, the incidence of cancer of the small intestine is 2/100,000 inhabitants, with a mean age at diagnosis of 69 years.<sup>2</sup> An increased risk of small bowel cancer is seen in individuals with Crohn's disease, familial adenomatous polyposis (FAP), or hereditary nonpolyposis colorectal cancer (HNPCC), but even in these conditions the dominant cancer risk for the patient is that of the colorectum.<sup>2,3</sup> Adenocarcinoma, which accounts for about half of the malignancies of the small intestine, is the most common histologic type, followed by

carcinoid tumor, lymphoma, and small intestinal stromal tumors. Tumor location differs between the carcinomas and the carcinoid tumors, with 40–50% of the adenocarcinomas affecting the duodenum and 80–90% of the carcinoid tumors arising in the ileum.<sup>1,2</sup> The reason for the low incidence of small bowel carcinoma compared to carcinoma of the colorectum, despite similarities in tissue structure and carcinogen exposure, is largely unknown. Differences in transit time, dilution of the intestinal contents, immunologic function, level of apoptosis, and amount of bacteria have been suggested as possible explanations for the rareness of cancer of the small intestine.<sup>1,2</sup> Cancers of the small and the large intestine share epidemiologic features, such as an increased incidence in the Western world and possible associations with dietary factors, smoking, and alcohol use.<sup>2,4</sup> Furthermore, the risk of colorectal carcinoma is increased following carcinoma of the small intestine and vice versa, indicating a common mechanism for these tumor types.<sup>5</sup> Similar to colorectal carcinomas, cancer of the small intestine develop from adenomas through an adenoma/dysplasia-carcinoma sequence, characterized by an accumulation of multiple genetic alterations.<sup>6–12</sup> Mutations of *KRAS* and LOH at 17p as well as increased expression of the p53 tumor suppressor protein, which are among the most frequently detected genetic alterations in colorectal carcinomas, have been reported to occur in about 30–60% of the carcinomas of the small intestine.<sup>6,8–12</sup> Increased transcriptional activation through inactivating mutations in the *APC*-gene or through oncogenic activation of  *$\beta$ -catenin* plays a central role in colorectal tumorigenesis. Allelic loss of 5q or mutations of the *APC/ $\beta$ -catenin* pathway have been reported in 0–60% of the carcinomas of the small intestine, and thus seem to be less common in this tumor type than in colorectal carcinoma.<sup>6,8–12</sup> About 15% of colorectal carcinomas are characterized by microsatellite instability (MSI), which reflects a defective DNA mismatch repair (MMR) system. Such MMR defects may result either from a germline MMR gene mutation, the majority of which affect *MLH1* or *MSH2*, or from somatic MMR gene inactivation, most commonly through epigenetic silencing via methylation of the *MLH1* promoter. Data on MSI in small bowel adenocarcinomas are limited, but MSI has been reported at an overall frequency of 20%.<sup>6,9,10,12–14</sup> Colorectal tumors with defective MMR frequently show somatic alterations in repetitive DNA tracts of several genes involved in growth control, apoptosis, and DNA-repair.<sup>15</sup> Only a few investigations regarding the occurrence of mutations in such repeat-containing genes have been performed in carcinomas of the small intestine, with frameshift muta-

tions in the *TGF $\beta$ RII* gene detected in a subset of small bowel carcinomas with MSI but at a lower frequency than in colorectal carcinomas of the MSI phenotype.<sup>6,8–10</sup>

An inherited MMR defect causes HNPCC, an autosomal dominant syndrome that affects about 1/1000 individuals and confers an increased risk of several types of cancer at a young age and a tendency to develop multiple primary tumors. Germline MMR gene mutations, most commonly affecting *MLH1* or *MSH2*, are identified in approximately 60–80% of the HNPCC patients.<sup>16,17</sup> Mutation carriers run an 80–90% lifetime risk of developing an HNPCC-associated cancer, most commonly affecting the colorectum, endometrium, ovaries, urinary tract, and small intestine. Although the risk of cancer of the small intestine in HNPCC patients is increased 25- to 100-fold compared to the general population, the corresponding lifetime risk is estimated to be 1–4%.<sup>3,16,17</sup> To investigate the involvement of MMR in the development of cancer of the small intestine, we assessed MSI and immunohistochemical expression of *MLH1* and *MSH2* in a population-based series of 90 small bowel adenocarcinomas and in a partly overlapping series of 43 cancers of the small intestine diagnosed before age 60 years.

## MATERIALS AND METHODS

### Patient Material

Two partly overlapping tumor sets were utilized; 1) a population-based series was studied to determine the overall frequency of defective MMR in adenocarcinomas of the small intestine and 2) a sample set containing all available tumors from patients younger than 60 years at diagnosis was studied to determine the contribution of HNPCC to the development of cancer of the small intestine in younger patients. Individuals diagnosed with cancer of the small bowel during 1958–1999 were identified in the population-based cancer registry in the southern Swedish health care region (currently 1.5 million inhabitants). The registry contains about 300,000 tumors and has been determined to cover at least 96% of all cancers diagnosed, with pathology confirmation for 98% of the cases. The original histopathologic reports were retrieved and new routine sections were stained with hematoxylin and eosin and re-evaluated by a gastrointestinal pathologist (B.H) to confirm the diagnosis, to verify a primary tumor origin within the small intestine, and to ascertain that representative tumor tissue was available in the tumor block. A primary tumor origin within the small intestine was defined as the presence of mucosal dysplasia or of an adenoma-carcinoma transition and, on autopsy cases, no evidence of other tumors. Patients with HNPCC, FAP, or inflammatory bowel dis-

ease were not excluded. Neither data on family history of cancer nor blood samples for mutation analysis were available. Ethical approval for the study was obtained from the ethics committee at Lund University.

### Series I

Between 1989 and 1999, 149 adenocarcinomas of the small intestine were diagnosed. We successfully retrieved 130 paraffin embedded tumor blocks. Of these 41 were excluded, 33 because a primary tumor origin within the small intestine could not be established, 6 because of autolysis or lack of MSI results, and 2 cases because of adenocarcinomas at other sites (one case with a hepatobiliary cancer and another case with a colon carcinoma and a renal carcinoma) within two years of the diagnosis of small bowel carcinoma. The mean age at diagnosis in the whole series was 69.4 years (range, 21–90 years) and among the 89 cases analyzed 67.6 years (range, 21–89 years). The male:female ratio was 1:1. Tumor location was duodenum in 46 tumors (52%), jejunum or ileum in 33 tumors (37%), and an unspecified site within the small bowel in 10 tumors (11%).

### Series II

We extended the study to include all individuals diagnosed with adenocarcinomas of the small intestine before age 60 years during the period 1958–1989, a total of 54 individuals. Of these, 20 tumor blocks were not possible to locate, 2 cases could not be confirmed to be primary within the small intestine, MSI analysis failed in 11 tumors, and 2 cases were excluded because of a diagnosis of adenocarcinoma within two years of the small intestinal carcinoma, which left 19 successfully analyzed samples. The total series (combining the 24 patients from the population-based series and the 19 patients in the extended study) thus included 43 patients diagnosed before age 60. The mean age among the cases analyzed was 49.6 years (range, 21–59 years), the male to female ratio was 1.4:1, and the tumor location was the duodenum in 15 tumors (35%), the jejunum/ileum in 25 tumors (58%), and an unspecified site within the small bowel in 3 cases (7%).

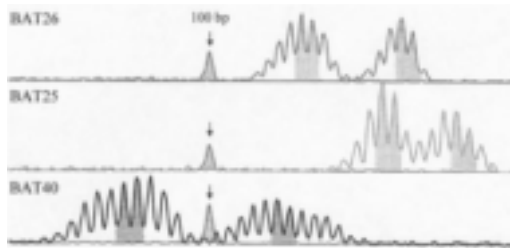
### Microsatellite Analysis

DNA was extracted from  $3 \times 10 \mu\text{m}$  sections of formalin-fixed, paraffin embedded tissue through incubation of the samples in ethylene diamene tetraacetic acid (EDTA)-Tris-buffer with proteinase K at 65 °C for at least two hours, followed by boiling, centrifugation, and removal of the aqueous phase, which was stored at 4 °C. The MSI status of the tumors was established using the mononucleotide markers BAT25, BAT26 and

BAT40 and the dinucleotide marker BAT34C4. The MSI-markers used herein are all among those recommended in the National Cancer Institute reference panel for MSI analysis and have been shown to assess MSI with high accuracy.<sup>18</sup> The markers BAT25, BAT26 and BAT34 are quasi-monomorphic (minor inter-individual size variations), with an allelic size variation not exceeding two nucleotides.<sup>19,20</sup> For the polymorphic marker BAT40, large size variations (–6 to –16 nucleotides from the most frequent allele) are rare. Therefore, these markers can be used to determine MSI status reliably even in the absence of normal control tissue. The sequences of the primers used were, for BAT25: 5'-TCGCCTCCAAGAATGTAAGT-3' (forward) and 5'-TCTGCATTTTAACTATGGCTC-3' (reverse); for BAT26: 5'-TGACTACTTTTGACTTCAGCC-3' (forward) and 5'-AACCATTCACATTTTAAACCC-3' (reverse); for BAT40: 5'-ACAACCCTGCTTTTGTTCCT-3' (forward) and 5'-GTAGAGCAAGACCACCTTG-3' (reverse); and for BAT34C4: 5'-ACCCTGGAGGATTTCATCTC-3' (forward) and 5'-AACAAAGCGAGACCAGTCT-3' (reverse). The markers were fluorescently-labelled as follows: TET<sup>TM</sup> (green) for BAT 25, 6-FAM<sup>TM</sup> (blue) for BAT 26 and BAT34C4, and HEX<sup>TM</sup> (yellow) for BAT 40. The DNA microsatellite sequences were amplified by polymerase chain reaction (PCR) according to the following program: 94 °C for 7 minutes, 10 × (94 °C for 15 seconds, 45 °C [BAT 25]/50 °C [other markers] for 15 seconds, and 72 °C for 15 seconds), 23 × (89 °C for 15 seconds, 45 °C/50 °C for 15 seconds, and 72 °C for 15 seconds), 72 °C for 7 minutes, and a final cooling step at 4 °C. Then, 0.5–2  $\mu\text{L}$  PCR product was mixed with 12  $\mu\text{L}$  deionized formamide (Hi-Di Formamide, Applied Biosystems) and 0.5  $\mu\text{L}$  TAMRA<sup>TM</sup> 500 Size Standard (Applied Biosystems, Foster City, CA), denatured at 95 °C for 3 minutes, and separated in Performance Optimized Polymer-4 (POP-4<sup>TM</sup>) on the ABI PRISM<sup>TM</sup> 310 Genetic Analyzer (Applied Biosystems). MSI was defined by the presence of extra peaks (Fig. 1). Data from at least three markers were required for the classification of tumors as microsatellite stable (MSS), with the exception of one tumor, which was recorded as MSS based on information from two loci only. The tumors were regarded as MSI-high (MSI-H) if at least two microsatellites showed instability and as MSI-low (MSI-L) if only one marker showed instability (Table 1).

### Immunohistochemistry

Immunohistochemical staining was performed using 4  $\mu\text{m}$  sections of formalin-fixed, paraffin embedded tissue, which were mounted on DAKO ChemMate Capillary Gap Microscope Slides (DAKO A/S BioTek Solutions, Glostrup, Denmark) and dried at room temperature overnight followed by incubation at 60 °C for



**FIGURE 1.** Microsatellite instability (MSI) analysis of an adenocarcinoma (Patient X139) showing MSI for the markers BAT26, BAT25, and BAT40. bp; base pairs.

one to two hours. The tissue sections were deparaffinized in xylol and rehydrated through descending concentrations of alcohol. Antigen retrieval was achieved by microwave-treatment in 1 mM EDTA, pH 9.0, at 900 W for 8 minutes followed by 15 minutes at 350 W. The slides were then allowed to cool for at least 20 minutes in EDTA solution. Immunohistochemical staining was performed in an automated immunostainer (TechMate 500 Plus, DAKO), according to the manufacturers' instructions. The main steps were as follows: mouse monoclonal immunoglobulin G antibodies to MLH1 (clone G168-15, dilution 1:100, PharMingen, San Diego, CA) or MSH2 (clone FE-11, dilution 1:100, Oncogene Research Products, Boston, MA) were applied and the sections were incubated at room temperature for 25 minutes, followed by incubation with biotinylated anti-mouse antibody (DAKO) for 25 minutes. Endogenous peroxidase activity was blocked in 3% H<sub>2</sub>O<sub>2</sub> for 3 × 2.5 minutes, followed by incubation with streptavidin-horseradish peroxidase for 25 minutes. Finally, the tissue sections were stained with diaminobenzidine, counterstained with hematoxylin, dehydrated in ascending concentrations of alcohol, and mounted. After each step, the sections were rinsed in Tris buffered saline, pH 7.6, and Triton X-100. To block nonspecific protein binding, bovine serum albumin was added to the buffer before and after the antibody incubation steps. The authors (M.P., K.E., and M.N.), who were blinded regarding the MSI status and the clinicopathologic data, independently evaluated all stained sections. Tumors from HNPCC patients with known germline mutations in *MSH2* and *MLH1*, respectively, were included in each staining round and served as controls. Sections without nuclear staining in the tumor cells, in the presence of normal nuclear staining in lymphocytes and normal epithelial or stromal cells in the same section, were considered to have a lost expression of the respective MMR protein (Fig. 2).

## RESULTS

### MSI Analysis

In the population-based series, MSI data were obtained from 89 tumors and revealed phenotypes that were MSS in 73 tumors (82%), MSI-H in 12 tumors, and MSI-L in 4 tumors, resulting in total MSI frequency of 18% (Table 1). Among the young (aged < 60 years) patients, 10/43 (23%) tumors displayed MSI, with 6 tumors being MSI-H and 4 MSI-L.

### Immunohistochemistry

All tumor samples were immunohistochemically stained using antibodies against the MMR-proteins MLH1 and MSH2. In the population-based series, 6 of the 73 MSS tumors could not be evaluated for MLH1 due to poor staining quality. Of the 67 evaluable MSS tumors, 2 showed loss of MLH1, whereas all MSS tumors showed normal expression of MSH2. Among the 16 MSI tumors, 15 were evaluable and 7 showed loss of MLH1, whereas MSH2 was expressed in all MSI tumors.

Among the 33 MSS tumors from the younger patients in the second series, one tumor showed loss of MLH1, whereas MSH2 protein expression was detected in all tumors. Among the 10 MSI tumors in the second series, loss of MLH1 was found in 3 tumors and loss of MSH2 in 3, and 4 tumors (3 MSI-L and 1 MSI-H) showed normal expression of both proteins.

In summary, loss of expression was found in 2/86 MSS tumors, in 1/6 MSI-L tumors, and in 10/15 evaluable MSI-H tumors. Among tumors with MSI and/or immunohistochemical MMR protein loss, the male:female ratio was 1:1.4, and the tumor location was the duodenum in 10 cases, the jejunum or ileum in 12 cases, and an unspecified site within the small bowel in two cases (Table 1).

## DISCUSSION

Cancers of the large and small intestine share many etiological factors and several of the somatic mutations characteristic of the adenoma/dysplasia-carcinoma sequence, albeit with variation in the absolute frequencies of the different mutations.<sup>5-12</sup> A role for defective MMR in the tumorigenesis of small intestinal carcinoma has been established through the increased risk of such tumors in HNPCC patients as well as in MMR deficient mice.<sup>3,16,17,21-23</sup> We have in a population-based study shown MSI in 16/89 (18%) cancers of the small intestine, with 12 tumors being MSI-H and 4 MSI-L. Previous studies of MSI in cancer of the small intestine have been small and have included many young patients but have shown an overall MSI frequency of 20%.<sup>6,8-12</sup> Taken together, the results sug-

**TABLE 1**  
**Tumors with MSI and/or Immunohistochemical MMR Protein Loss**

Series	Patient No	BAT25	BAT26	BAT34	BAT40	MSI	MLH1	MSH2	Age	Gender	Tumor location
I	X68	+	0	+	+	H	IC	IC	85	f	Jejunum
	X12	+	+	-	0	H	-	+	74	f	Jejunum
	X33	+	+	0	+	H	-	+	80	m	Duodenum
	X88	+	-	+	0	H	-	+	69	f	Jejunum
	X91	+	+	0	+	H	-	+	54	m	NOS
	X97	+	+	-	+	H	-	+	74	f	Duodenum
	X99	+	0	0	+	H	-	+	63	m	Jejunum
	X15	+	+	0	+	H	+	+	66	m	Duodenum
	X42	+	+	0	+	H	+	+	48	f	Ileum
	X74	+	0	+	0	H	+	+	63	m	Duodenum
	X98	-	+	-	+	H	+	+	65	f	NOS
	X79	+	-	-	+	H	+	+	77	m	Duodenum
	X65	-	+	-	-	L	-	+	57	m	Duodenum
	X57	-	-	-	+	L	+	+	77	f	Duodenum
	X90	+	0	-	-	L	+	+	65	f	Jejunum
	X18	-	-	-	+	L	+	+	57	f	Ileum
	X53	-	-	0	-	MSS	-	+	52	f	Duodenum
	X95	-	-	-	-	MSS	-	+	71	f	Duodenum
	II	X138	+	+	+	0	H	-	+	58	f
X123		0	0	+	+	H	+	-	45	m	Duodenum
X134		+	0	+	0	H	+	-	54	f	Jejunum/ileum
X139		+	+	+	0	H	+	-	56	f	Jejunum/ileum
X115		-	0	+	-	L	+	+	57	m	Jejunum
X131		-	-	+	-	L	+	+	57	m	Ileum

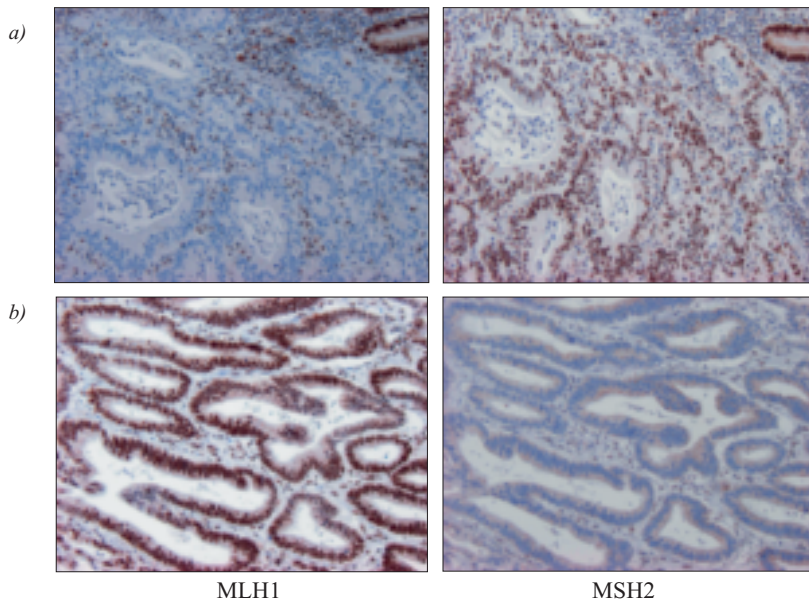
MSI: microsatellite instability; +: retained mismatch repair protein expression; -: loss of mismatch repair protein expression; 0: no data available; H: MSI-high; L: MSI-low; IC: inconclusive; +: retained MMR protein expression -; loss of MMR protein expression f: female; m: male; NOS: not otherwise specified; MSS: microsatellite stable. Shading: individual below age 60 years at diagnosis.

gest that MSI occurs at about the same frequencies in adenocarcinomas of the small and the large intestine.

In order to delineate the contribution of defective MMR in tumors from younger patients and as a possible indication of HNPCC, we extended the study and showed MSI in 23% of the tumors from patients diagnosed before age 60 years. Approximately 20% of the colorectal carcinomas with MSI are estimated to represent HNPCC-associated tumors and are thus associated with germline mutations, most commonly affecting *MLH1* or *MSH2*. In line with clinicopathologic data on HNPCC-associated colorectal carcinoma, HNPCC patients with carcinomas of the small intestine have a lower age of onset, a higher male to female ratio, a high incidence of metachronous tumors, and a different site distribution within the small bowel (an even distribution of tumors in HNPCC, compared to a predilection for tumors in the duodenum among the sporadic cases).

Several studies have shown a good correlation between MSI status and MMR immunoreactivity in colorectal carcinoma, with frequent loss of MMR protein expression in MSI tumors and retained expression in MSS tumors, although occasional such tumors show loss of expression.<sup>24-26</sup> Hence, a combination of

these techniques predicts MMR status with high accuracy. Whereas loss of expression of *MSH2* occurs almost exclusively in HNPCC-patients, loss of *MLH1* expression occurs in the majority of sporadic MSI cancers due to epigenetic silencing through promoter hypermethylation.<sup>24-27</sup> In the population based series, we identified loss of immunoreactivity for *MLH1* in 7/15 evaluable MSI tumors and in 2/67 MSS tumors. These findings suggest that *MLH1* silencing is, like in other types of gastrointestinal tumors, the main mechanism behind defective MMR in cancers of the small intestine, but it also indicates that other mechanisms or other genes may be causative in the remaining MSI tumors. Normal immunohistochemical staining patterns and lack of MMR gene mutations have previously been reported in the majority of MSI-L tumors, indicating that these different degrees of MSI also reflect separate tumorigenic mechanisms.<sup>27,28</sup> Low-level MSI can be shown in a large fraction of non-MSI tumors if multiple markers are studied, which makes the definition as well as the qualitative importance of MSI-L tumors uncertain.<sup>29</sup> MSI-L tumors with MSI primarily affecting mononucleotide repeats have been associated with mutations in the *MSH6* gene, which was not investigated in the current study.<sup>30</sup> Loss of



**FIGURE 2.** Immunohistochemical staining for MLH1 and MSH2 in tumors from a) Patient X138 and b) Patient X100. Tumor X138 shows loss of expression for MLH1 and retained expression of MSH2, whereas tumor X100 shows retained expression for MLH1 and loss of expression for MSH2.

MLH1 protein expression was observed in one of the MSI-L tumors in the current study. Since we analyzed a limited number of MSI markers, some of the MSI-L tumors might indeed represent MSI-H cases, and, likewise, the two MSS tumors with loss of MMR expression could be false negative MSI tumors (Table 1). Among the young (aged < 60 years) patients, immunohistochemical loss was found in 6/10 MSI tumors and affected MLH1 and MSH2 at equal frequencies, suggesting that HNPCC may be the underlying cause of defective MMR in at least some of these tumors.

In summary, we have in a population-based series of carcinomas of the small intestine shown MSI in 18% of the tumors; the contribution of defective MMR to the carcinogenesis in the small intestine is thus similar to that observed in the large intestine. However, whereas *MLH1* is found to be defective in the vast majority of MSI colorectal carcinomas, the current results indicate that only about half of the MSI tumors show loss of MLH1 expression. In patients with carcinomas of the small intestine before age 60 years, MSI was detected in 23% of the tumors, and, in these tumors, immunohistochemistry revealed loss of MLH1 and MSH2 at about equal frequencies. Since somatic mutations are rare in *MSH2*, these findings suggest that a subset of these tumors are HNPCC-associated. Thus, in patients with early onset small bowel carcinoma and/or a family history suggesting HNPCC, a combined analysis of MSI and immunohistochemical

staining of MLH1 and MSH2 may be a valuable diagnostic tool.

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### **Microsatellite Instability and Expression of MLH1 and MSH2 in Carcinomas of the Small Intestine**

**W**e read the article by Planck et al.<sup>1</sup> with great interest. Microsatellite instability (MSI) has been reported previously in 15–45% of small bowel carcinomas (SBCs).<sup>2,3</sup> Fifteen percent of unselected colorectal carcinomas (CRCs) show high MSI (MSI-H). A loss of MLH1 expression can be observed in 70% of sporadic MSI-H colorectal carcinomas.

It is very surprising that < 50% of the MSI-H tumors in the population-based group and 60% of the MSI-H

tumors in the early-onset group (age < 60 years) exhibited loss of MLH1 or MSH2. There are two possible reasons for this result. First, a technical reason may be the definition and assessment of MSI-H and low MSI (MSI-L) by the investigators. The choice of MSI markers was not concordant with the Bethesda reference panel.<sup>4</sup> Moreover, the authors restricted the MSI analysis to the tumor. Normal tissue was not examined. If the analysis had been restricted to the most monomorphic marker (*BAT26*), then 10 tumors displayed instability in *BAT26*. Seven of those tumors showed loss of MLH1 or MSH2 (70%), which is comparable to the findings in colorectal carcinoma. We are especially concerned about the use of *BAT40* without analysis of normal tissue. *BAT40* is not as monomorphic compared with *BAT26*, which may significantly hamper the interpretation of MSI results. Three tumors without shift of *BAT26* and normal mismatch-repair (MMR) protein expression were classified as MSI-L (only *BAT40* shifts) or MSI-H (*BAT40* and *BAT25*). Unfortunately, the authors did not define their interpretation of instability for each marker. We question the instability assessment of *BAT40* in these three tumors, which could be classified as false-positive. This might have been avoided by analysis of normal tissue as a control. Moreover, two tumors showed loss of MLH1 but displayed microsatellite stability (MSS) status, which is a very unusual finding. Unfortunately, this was not discussed by the authors. It is possible that these tumors displayed a false-negative MLH1 reaction (internal control?) or that microsatellite analysis detected a false-negative MSS status, which may occur especially in mucinous carcinomas (low tumor cellularity) or may have been caused by the technical limitations of the study, as discussed above. Therefore, we conclude that the assessment of MSI in this study has some noteworthy limitations and that assessment of MSI should follow the Bethesda guidelines, using the reference panel and examination of tumor and normal tissue.

The second possible reason for the relatively low rate (50–60%) of loss of MMR protein expression is that other MMR genes, such as *MSH6*, *PMS2*, or *MLH3*, may contribute significantly to MSI-H SBC. Regarding this hypothesis, knowledge of family data would have been of great interest to identify hereditary nonpolyposis colorectal carcinoma (HNPCC)-related SBC. Moreover, patients with a history of previous malignancies within 2 years were excluded from the study. The development of multiple tumors is characteristic for HNPCC; therefore, the inclusion of patients with previous malignancies may have resulted in a higher incidence of MSI-H SBC and a higher rate of tumors with MMR protein loss.

Another point to be discussed is that the authors identified 18% MSI-H SBC in an unselected, popula-

tion-based series and identified 23% MSI-H SBC in a series of patients with early-onset SBC (< 60%). They conclude that MSI and immunohistochemical analysis of SBC may be a valuable tool in patients with early-onset SBC. However, there was only a slight improvement of the MSI incidence when the analysis was restricted to patients with early-onset SBC. Therefore, we conclude that a restriction to patients with early-onset SBC is not appropriate when a cut-off age of 60 years is used. The median age of patients with HNPCC-related SBC is 49 years (range, 25–88 years).<sup>5</sup>

Therefore, sensitive and specific algorithms for MSI testing of SBC, including family history of HNPCC and age at diagnosis of SBC, still have to be defined to identify HNPCC-related SBC. In contrast, it is possible to screen all SBC for MSI to identify HNPCC, because SBC is a rare condition, and HNPCC may be responsible for a substantial proportion of SBC. To date, there still are limited data on this topic, and further studies are necessary.

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## Author Reply

**W**e thank Schulmann et al. for the valuable comments related to our article.<sup>1</sup> We have rechecked the microsatellite instability (MSI) status and performed additional immunostainings in our material and would like to comment on the questions raised.

The objective of our study was to evaluate the frequency of defective mismatch-repair (MMR) in a population-based series of small bowel carcinomas in the Southern Sweden health care region, 1989–1999 (Series I), and in patients who were diagnosed before age 60 (Series II). Because the paraffin-embedded tumor blocks were collected from different pathology departments, and the majority of the patients were diseased, data on family history of cancer were not available. For practical reasons, only tumor-containing blocks were available; therefore, we chose to use mononucleotide markers for the MSI analysis, because these markers can be interpreted without the need for matching normal tissue. However, we agree that an MSI classification based on the instability of *BAT40* is suboptimal, although normal allelic size variations as large as those classified as unstable in our material are rare.<sup>2</sup> Hence, we cannot rule out the possibility that these two tumors represent false-positive results. Because data on MSI and immunostaining in carcinoma of the small intestine are scarce, it is not known which markers detect MSI with the greatest sensitivity in this tumor type. The sensitivity of the MSI markers depends on the tumor type, as demonstrated, e.g., for colorectal carcinoma and endometrial carcinoma. Furthermore, even if all 4 tumors with low MSI (MSI-L; classified based on data from 3 MSI markers, of which only one was positive) in the population-based series had been excluded, only 7 of 11 tumors (65%) with high MSI (MSI-H) showed loss of MLH1 or MSH2.

We do not believe that the MSI-H tumors with retained immunostaining represented false negative MLH1 staining; immunostaining was retained in the stromal components and in the tumor-infiltrating lymphocytes of all these tumors. We routinely perform MSI and MMR protein immunostaining to identify hereditary nonpolyposis colorectal cancer in patients with colorectal carcinoma and have previously experienced a high degree of correlation between the MSI and MMR protein immunostaining.

The two tumors with microsatellite stability (MSS) that showed a loss of MLH1 staining likely represent false negative MSI tumors, and although it is a rare finding, loss of MMR protein expression in MSS tu-

mors has been described by several authors and often has been associated with suboptimal archival tumor tissue.<sup>3</sup>

To characterize the genetic defect behind the MSI tumors with retained expression of MLH1 and MSH2, we have immunostained the 23 evaluable tumors (described in Table 1 of our recent article<sup>1</sup>) for the MMR protein MSH6. In the population-based series (Series I), one tumor (X42) showed loss of MSH6 staining, and one tumor (X115) among the young patients (Series II) also showed loss of MSH6 alone. In addition, loss of MSH6 was (as expected) observed in three tumors (X123, X134, and X139) with loss of MSH2 staining. Hence, after inclusion of MSH6 staining, loss of MMR protein staining was found in 7 of 15 MSI tumors in the population-based series and in 8 of 10 tumors among individuals younger than age 60 years.

In summary, adding immunostaining for MSH6 indeed may explain an additional two discordant (MSI and retained immunostaining for MLH1 and MSH2) tumors. However, in the whole material retained staining for the MMR proteins MLH1, MSH2, and MSH6 still was present in 10 of 21 MSI tumors. Therefore, these findings suggest that other mechanisms may cause a subset of the MMR-defective carcinomas of the small bowel.

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