Deficient DNA Mismatch Repair Is Common in Lynch Syndrome-Associated Colorectal Adenomas

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Lynch syndrome is caused by germline mutations in DNA mismatch repair (MMR) genes. Both microsatellite instability (MSI) testing and immunohistochemical analyses (IHC) of colon cancers are valuable diagnostic strategies for Lynch syndrome. We sought to determine whether these markers of MMR deficiency were also detectable in pre-cancerous colorectal adenomas. Fifteen subjects with a germline MMR gene mutation who had 44 adenomas removed during surveillance colonoscopy were identified. MSI testing and IHC for MLH1, MSH2, and MSH6 were performed. MSI was detected in 23 adenomas. There was a significant association between MSI and high-grade dysplasia ($P = 0.006$) and distal location ($P = 0.0008$). Loss of MMR protein by IHC was detected in 31 adenomas. A significant association was observed between loss of staining by IHC and high-grade dysplasia ($P = 0.04$). Among the 40 adenomas in which both MSI tests and IHC were performed, the presence of a germline mutation correlated with an abnormal MSI result in 58% of cases, an abnormal IHC result in 70% of cases, and either an abnormal MSI or IHC result in 73% of cases. The combination of MSI and IHC testing in colorectal adenomas is a sensitive screen for the detection of Lynch syndrome and may be particularly useful when Lynch syndrome is suspected and adenomatous polyps are the only tissues available for analysis. (*J Mol Diagn* 2009, 11:238–247; DOI: 10.2353/jmoldx.2009.080142)

Lynch syndrome, also known as hereditary nonpolyposis colorectal cancer, is an autosomal dominant disorder that accounts for 3% to 5% of all colorectal cancers.¹ Lynch syndrome is characterized by high penetrance, early-onset colorectal cancer and an increased risk of certain extra-colonic cancers, including tumors of the endome-

trium, stomach, small bowel, ovary, hepatobiliary tract, renal pelvis, and ureter.^{2,3} Germline mutations of DNA mismatch repair (MMR) genes, most commonly *MLH1*, *MSH2*, or less frequently *MSH6* and *PMS2*, underlie the majority of cases of Lynch syndrome.^{4–9} Mutations in these genes impair the function of MMR proteins, which normally recognize and repair mismatched nucleotides and insertion/deletion loops caused by slippage of DNA polymerase.^{10,11} In tumors that develop due to defective DNA mismatch repair, repetitive DNA sequences known as microsatellites tend to undergo a high level of genetic alteration, resulting in microsatellite instability (MSI).^{12–14} MSI can be identified in greater than 90% of colorectal cancers that arise in individuals with Lynch syndrome, whereas in sporadic colorectal cancer it occurs in 15% of cases, typically from silencing of the *MLH1* gene by promoter hypermethylation.¹⁵ Microsatellite analysis has therefore been proposed as a useful diagnostic tool to screen for Lynch syndrome.^{16,17} In addition, immunohistochemical analysis (IHC) for the four MMR proteins is a complementary approach that can pinpoint the specific gene most likely to be mutated.^{18–21}

The diagnosis of Lynch syndrome can be difficult to make because family history information is often incomplete and there is no characteristic clinical phenotype such as diffuse polyposis. Nevertheless, its early recognition is essential to identify patients at high risk who will require intensive cancer surveillance. In Lynch syndrome, carcinogenesis proceeds through the adenoma-carcinoma sequence, albeit at a more rapid pace. A significant patient survival advantage and reduction in the incidence of colorectal tumors has been observed following colonoscopic screening and polypectomy.²² Although the number of polyps in Lynch patients appears to be similar to the general population, the polyps are more likely to occur at a younger age, have a predilection for the proximal colon, be larger, display villous features or high-grade dysplasia, and most importantly, grow rapidly and progress to invasive cancer in less than 3 years.^{23–25}

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The recognition of hereditary colon cancer syndromes and Lynch syndrome, in particular, is increasing in the population. Many individuals with suspected Lynch syndrome now undergo routine colonoscopic screening with polypectomy. In such a scenario, there is no colon cancer tissue available for MSI and IHC testing. The present study was undertaken to test the hypothesis that MSI testing and IHC analysis in pre-cancerous colorectal adenomas instead of colorectal cancers may be an alternative approach to screen for Lynch syndrome.

Methods

Patients

Carriers of a known germline mismatch repair gene mutation referred to the Gastrointestinal Cancer Genetics Clinic at the Massachusetts General Hospital were identified. Those who had polyps endoscopically removed during surveillance examinations between January 1997 and December 2007 were selected for further analysis. Whenever available, hyperplastic polyps were also collected. Genetic counseling and testing were performed as part of routine clinical care. Sequencing of the *MLH1*, *MSH2* and *MSH6* genes was performed in Clinical Laboratory Improvement Amendments-certified laboratories as part of routine clinical care and included sequencing of all exons and exon-intron boundaries as well as gene rearrangement analysis. All mutations were determined to be deleterious with the exception of a variant of uncertain significance in the *MLH1* gene in case 2 (H718P). Endoscopy and pathology reports from all patients enrolled were reviewed, and the location, size, and histopathological features of all polyps were recorded. The cecum, ascending colon, and transverse colon were regarded as the proximal or right colon, while the descending colon, sigmoid, and rectum were referred to as the distal or left colon. The degree of dysplasia and the presence of villous architecture were independently evaluated by M.M.-K. and A.J.I. This protocol was approved by the institutional review board of the Massachusetts General Hospital.

Analysis of MSI

MSI assays were performed on microdissected DNA, extracted using the Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN), from paraffin-embedded tissue blocks. Primer sets comprised the five reference panel markers recommended by the National Cancer Institute, with 5' phosphoramidite fluorescent labeling of forward primers as follows: BAT-25 (NED), BAT-26 (6-FAM), D5S346 (VIC), D17S250 (6-FAM), and D2S123 (VIC).¹³ The primer sequences for D2S123 were 5'-AA-CATTGCTGGAAGTTCTGG-3' (forward) and 5'-GTGTCT-TGACTTCCACCTATGGGACTG-3' (reverse). Primer sequences for the remaining loci were identical to those previously described except that a 5' GTGTCTT sequence was added to each reverse primer to facilitate non-template adenylation of the 3' end of the forward

strand. PCR amplifications were performed in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). PCR was conducted in a total volume of 20 μ L containing 1X Platinum TaqPCR buffer, 200 μ mol/L dNTPs, 2.0 mmol/L MgCl₂, 0.4 μ mol/L primers, and 1.0 U of Platinum Taq polymerase (Invitrogen, Carlsbad, CA), with 40 ng of genomic DNA, using the following conditions: initial denaturation at 94°C for 5 minutes, followed by 38 cycles of denaturation at 94°C for 30 seconds, annealing at either 50°C or 55°C for 30 seconds, and primer extension at 72°C for 30 seconds. The final extension step was performed at 72°C for 10 minutes. PCR products were pooled and fractionated by size using an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) and microsatellite status was analyzed using GeneMapper software. Only cases showing unequivocally distinct additional peaks or shifts in adenoma DNA in comparison with normal DNA were recorded and classified as MSI. The microsatellite status of each sample was determined based on the percentage of unstable loci. The status was defined as MSI-high (MSI-H) when two or more of five markers displayed instability and as MSI-low (MSI-L) when 1 marker exhibited instability. A sample was classified as microsatellite stable (MSS) when no MSI was found.

IHC Procedure

Staining of MMR proteins was performed using the BenchMark XT automated tissue staining system (Ventana Medical Systems, Inc., Tucson, AZ) using validated protocols. Briefly, slides were deparaffinized and endogenous peroxidase activity was blocked by incubation with 3% H₂O₂. Heat-induced antigen retrieval was performed using the Ventana CC1 mild reagent (Ventana Medical Systems), a combination of EDTA and boric acid in Tris buffer, and the process was performed for 30 to 60 minutes. After treatment with 10% normal goat serum to block nonspecific protein binding, prediluted primary mouse monoclonal antibodies against MLH1 (CellMarque, Rocklin, CA), MSH2 (Ventana Medical Systems), or MSH6 (B.D. Transduction Laboratories, Franklin, NJ, 1:50 dilution) were applied, followed by incubation with horseradish peroxidase-conjugated multimer antibody reagent (Igs; Ventana Medical Systems). The antigen-antibody reaction was visualized using diaminobenzidine as chromogen (UltraView, Ventana Medical Systems). Finally the slides were lightly counterstained with hematoxylin. Normal colonic crypt epithelium adjacent to the adenoma and lymphoid/stromal cells served as internal positive controls for staining. Adenomas that exhibited complete absence of nuclear staining in which adjacent normal mucosa or stromal/lymphoid cells showed intact nuclear staining were scored "negative" for expression of that protein.

Statistical Analysis

Continuous variables are expressed as median and range and categorical variables as absolute values or

rates. Differences with respect to categorical covariates were evaluated using the χ^2 test (overall or for trend) or Fisher's exact test on appropriate cross-tabulations. *P* values <0.05 were considered statistically significant. For the calculation, a statistical package (SPSS Inc, Chicago, IL) was used.

Results

Clinicopathological Features of Lynch Syndrome-Associated Colorectal Adenomas

Fifteen patients (10 males and five females) with a germline mutation in the *MLH1*, *MSH2* or *MSH6* gene and at least one adenoma removed during a surveillance colonoscopy were identified (Table 1). These 15 patients com-

prised 15 different kindreds. The median age at first polypectomy was 49 years (range, 39 to 68 years). Forty-four polyps were detected during 31 colonoscopies over a 10-year period from 1997 to 2007, resulting in a mean of 2.9 polyps per patient (range, 1 to 15) and 1.46 polyps per examination (range, 1 to 4). Thirty adenomas were from six carriers of an *MLH1* germline mutation, 11 adenomas were from seven subjects with an *MSH2* germline mutation, and three adenomas were from two patients with an *MSH6* germline mutation. During this time period, six patients developed one adenoma, four patients had two adenomas, and five patients were found to have three or more adenomas. Twelve patients (five with an *MLH1* germline mutation, five with an *MSH2* germline mutation, and two with an *MSH6* germline mutation) developed at least one Lynch-syndrome associated

Table 1. Clinicopathological Findings of Adenomas from Lynch Syndrome Patients

Case	Sex/Age (years)	Size (mm)	Histology	Dysplasia grade	Adenoma location	Mutated gene	Name of mutation	IHC results	MSI status
1	M/54	5	TA	LG	D	<i>MLH1</i>	1758insC	Loss	MSI-H
	56	4	TA	LG	D			Loss	MSI-H
2	F/49	3	TA	LG	P	<i>MLH1</i>	H718P	Preserved	MSS
	52	20	TA/HP	LG	P			Loss/Preserved	MSS
	56	10	TA	LG	P			Preserved	MSS
	57	4	TA/HP	LG	P			Loss/Preserved	ND
	58	10	TA/HP	LG	P			Preserved	MSS
3	M/39	8	TA	HG	P	<i>MLH1</i>	1024del16	Loss	MSI-H
	39	15	TA	LG	D			Loss	MSI-H
	40	9	TA	LG	P			Loss	MSS
	40	5	TA	LG	P			Loss	ND
4	M/45	8	TA	LG	D	<i>MLH1</i>	del exons1-6	Loss	MSI-H
	47	6	TA	LG	P			Preserved	MSS
	47	2	TA	LG	D			Loss	MSI-H
	47	2	TA	LG	P			Loss	MSS
	50	7	TA	LG	D			Loss	MSI-L
	50	7	TA	HG	P			Loss	MSI-H
	50	6	TA	HG	D			Loss	MSI-H
	50	6	TA	LG	D			Preserved	MSI-H
	50	22	TA	LG	D			Loss	MSI-L
	50	4	TVA	HG	P			Loss	MSI-H
	50	6	TA	LG	P			Loss	MSS
	50	7	TA	LG	P			Loss	MSI-H
	50	6	TA	HG	P			Loss	MSI-H
	50	6	TA	LG	P			Preserved	MSS
	54	7	TA	LG	D			Loss	ND
5	F/41	4	TA	LG	P	<i>MLH1</i>	Q62X	Loss	MSS
6	M/39	4	TA	LG	P	<i>MLH1</i>	1983insC	Loss	MSI-H
	39	8	TA	LG	P			Loss	MSS
	39	14	TA	HG	P			Loss	MSI-H
7	F/68	3	TA	LG	P	<i>MSH2</i>	2116delG	Loss	MSI-H
	68	6	TA	LG	P			Loss	MSI-H
	68	22	TA	HG	P			Loss	MSI-H
8	F/47	4	TA	LG	P	<i>MSH2</i>	229delAG	Preserved	MSS
9	M/48	3	TVA	LG	P	<i>MSH2</i>	388delCA	Loss	MSI-H
	48	8	TA	LG	D			Loss	MSI-H
10	M/47	4	TA	LG	P	<i>MSH2</i>	S743X	Preserved	MSS
11	M/67	6	TA	HG	D	<i>MSH2</i>	1687insT	Loss	MSI-H
12	M/49	4	TA	LG	P	<i>MSH2</i>	E48X	Preserved	MSS
13	F/47	4	TA	LG	D	<i>MSH2</i>	IVS5 + 3A>T	Preserved	ND
	49	12	TA	LG	P			Loss	MSI-H
14	M/47	4	TA	LG	P	<i>MSH6</i>	522delAG	Preserved	MSS
	47	7	TA	LG	P			Preserved	MSS
15	M/52	4	TA	LG	P	<i>MSH6</i>	C687X	Preserved	MSS

M, male; F, female; TA, tubular adenoma; HP, hyperplastic polyp; TVA, tubulo-villous adenoma; LG, low-grade; HG, high-grade; D, distal; P, proximal; IHC, immunohistochemistry; MSI, microsatellite instability; MSI-H, high frequency microsatellite instability; MSI-L, low frequency microsatellite instability; MSS, microsatellite stable.

carcinoma (13 colorectal, two endometrial, two duodenal, one bladder, and one gastric carcinoma), and four were diagnosed with two different primary tumors. In two of the 10 patients with a diagnosis of colorectal cancer, adenomatous polyps were diagnosed synchronously with a right-sided colon cancer. In the majority of cases, the adenomas were identified during surveillance colonoscopy.

The sites and pathological features of the 44 polyps are summarized in Table 2. These Lynch syndrome-associated adenomas were more commonly identified in the right colon, with 31 out of the 44 (70%) adenomas located proximal to the splenic flexure. Histopathological review confirmed the diagnosis of 39 tubular and two villous adenomas, and there were three mixed polyps that exhibited features of both tubular adenoma and hyperplastic polyp. The adenomas displayed a wide range of size with a mean of 7.2 mm (range, 2 to 22 mm). Sixteen of the 44 (36%) adenomas were smaller than 5 mm, 22 (50%) were between 5 and 10 mm, and six (14%) were larger than 10 mm. The majority of adenomas (82%) exhibited only low-grade dysplasia, but high-grade dysplasia was identified in 8 of the 44 adenomas. Of these eight, six were located in the right colon with a median size of 9.1 mm (range, 4 to 22 mm).

MSI Status in Adenomatous Polyps

Sufficient tissue was available for assessment of MSI in 40 of the 44 adenomas. MSI was detected in 23 of the 40 adenomas (58%). A high level of MSI (MSI-H), defined as instability in at least two of the five microsatellite markers analyzed, was found in 21 adenomas. This included 14 and seven adenomas from carriers of an *MLH1* or *MSH2* germline mutation, respectively. A low level of MSI (MSI-L), defined as instability in only one marker, was detected in two adenomas from one patient with an *MLH1* germline mutation. The remaining 17 adenomas (42%), including all adenomas from patients with an *MSH6* germline mutation, did not demonstrate any microsatellite alteration and were classified as MSS. When the data were analyzed per subject, the sensitivity of MSI analysis for the

detection of defective mismatch repair in Lynch syndrome-associated adenomas was 53%. Using MSI testing, eight out of fifteen patients (four with an *MLH1* and four with an *MSH2* gene mutation) were correctly identified as a carrier of an MMR gene mutation. As shown in Table 3, there was no significant association between MSI status and sex, age, or adenoma size. Although microsatellite instability was detected in 65% of the adenomas ≥ 5 mm compared with 43% of the adenomas < 5 mm, this difference was not statistically significant. Figure 1A depicts this distribution of the 40 adenomas by size and MSI status. Interestingly, whereas only 41% of the 29 proximally located adenomas showed MSI, all of the 11 distally located adenomas were MSI-H ($P = 0.0008$). Figure 1B illustrates the distribution of the 40 adenomas by location and MSI status. Finally, all eight adenomas with high-grade dysplasia exhibited MSI ($P = 0.006$).

IHC Analysis

IHC staining was performed on all 44 adenomas. Loss of MMR protein immunostaining, defined as complete absence of nuclear staining within the adenoma, was detected in 31 of the 44 (70%) cases. Of these 31 adenomas, 24 were from patients with a germline *MLH1* mutation and seven were from patients with a germline *MSH2* mutation. Staining was preserved in the three adenomas from two patients with an *MSH6* mutation. Among the 30 adenomas from carriers of an *MLH1* mutation, loss of MLH1 staining was observed in 24 (80%) adenomas. The median size of these adenomas was 7.6 mm (range, 2 to 22 mm), and the majority (15/24; 63%) were located proximal to the splenic flexure. Among the six adenomas from patients with a germline *MLH1* mutation with preserved MLH1 staining, all but one (83%) were located in the right colon with a median size of 6.8 mm (range, 3 to 10 mm). Loss of MSH2 staining was identified in 64% (7/11) of the adenomas from carriers of an *MSH2* mutation. The median size of these adenomas was 8.6 mm (range, 3 to 22 mm), and five out of seven were located in the right colon. The four adenomas with pre-

Table 2. Pathological Features of Lynch Syndrome-Associated Adenomas

	Adenomas (n = 44)
Location	
Proximal colon	31 (70%)
Distal colon	13 (30%)
Histologic type	
Tubular adenoma	39 (89%)
Villous adenoma	2 (4%)
Mixed adenomatous/hyperplastic polyp	3 (7%)
Size (mm)	
<5	16 (36%)
5–10	22 (50%)
>10	6 (14%)
Dysplasia	
Low-grade	36 (82%)
High-grade	8 (18%)

Table 3. Relationship between MSI Status and Clinicopathological Features of Adenomas

	n	MSI-H/ MSI-L	MSS	P value
Sex				
Male	30	19	11	0.2
Female	10	4	6	
Age				
<50	20	9	11	0.1
≥ 50	20	14	6	
Size (mm)				
<5	14	6	8	0.08
5–10	20	12	8	
>10	6	5	1	
Location				
Proximal colon	29	12	17	0.0008
Distal colon	11	11	0	
Dysplasia				
Low-grade	32	15	17	0.006
High-grade	8	8	0

Figure 1. A: Distribution of 40 Lynch syndrome associated-adenomas by size and MSI status. The mean size is indicated by the horizontal bar. **B:** Distribution of 40 Lynch syndrome associated-adenomas by location in the colon, MSI status, and genotype.

served MSH2 staining were from four different patients, and three were located in the right colon, all with a size of 4 mm. IHC analysis demonstrated a sensitivity of 67% when the analysis was performed on a per patient basis. Immunostaining for defective mismatch repair proteins in Lynch syndrome-associated adenomas identified the gene involved in ten out of fifteen patients (all six carriers of an *MLH1* mutation and four out of seven carriers of an *MSH2* mutation).

As shown in Table 4, no statistically significant correlation was found between sex, age or location with loss

Table 4. Relationship between IHC and Clinicopathological Features of Adenomas

	n	Loss	Preserved	P value
Sex				
Male	32	24	8	0.2
Female	12	7	5	
Age				
<50	22	14	8	0.3
≥50	22	17	5	
Size (mm)				
<5	16	9	7	0.04
5–10	22	16	6	
>10	6	6	0	
Location				
Proximal colon	31	20	11	0.1
Distal colon	13	11	2	
Dysplasia				
Low-grade	36	23	13	0.04
High-grade	8	8	0	

of immunostaining. However, adenomas located distally more frequently displayed loss of MMR protein staining. Among the 16 adenomas smaller than 5 mm, nine (56%) lost MMR protein expression compared with 16 of 22 (73%) adenomas between 5 and 10 mm, and all six adenomas larger than 10 mm ($P = 0.04$) (Figure 2A). Twenty of the 31 (65%) proximally located adenomas lost staining compared with 11 of the 13 (85%) distal adenomas, but this difference did not reach statistical significance. In Figure 2B, the distribution of all 44 adenomas by location and IHC results is shown. Finally, loss of staining was observed in all eight adenomas with high-grade dysplasia ($P = 0.04$). Different immunostaining patterns in independent adenomas from the same patient were found in 3 cases (one with an *MSH2* mutation and two with an *MLH1* mutation). Representative immunostaining patterns for adenomas are illustrated in Figure 3A.

Correlation of MSI and IHC Testing in Adenomas

There were 40 adenomas in which both MSI and IHC testing were performed. In these 40 cases, negative protein expression by IHC analysis was detected in 22 of the 23 adenomas that exhibited MSI. Specifically, 15/23 (65%) showed loss of MLH1 nuclear staining and 7/23 (30%) exhibited loss of MSH2 staining. The only ade-

Figure 2. A: Distribution of 44 Lynch syndrome associated-adenomas by size and IHC results. The mean size is indicated by the horizontal bar. **B:** Distribution of 44 Lynch syndrome associated-adenomas by location in the colon, IHC results, and genotype.

Figure 3. A: Representative cases of lost or preserved immunostaining of MMR proteins in adenomas from Lynch syndrome patients. **B:** Representative case of preserved MSH2 staining in a hyperplastic polyp from a Lynch syndrome patient.

noma with abnormal MSI but normal IHC results was a 6-mm sigmoid adenoma derived from a carrier of an *MLH1* germline mutation. Among the 17 adenomas that did not exhibit MSI, 11 (65%) showed intact staining of the MMR protein tested and six, from four different carriers of an *MLH1* germline mutation, had loss of expression of the MLH1 protein. All seven adenomas from carriers of an *MSH2* mutation in which IHC analysis demonstrated loss of MSH2 protein staining also exhibited MSI. Although no abnormalities in IHC or MSI testing were observed in the adenomas from *MSH6* gene mutation carriers, IHC and MSI testing correctly identified *MLH1* gene mutation carriers in 100% and 67% of cases, respectively, and *MSH2* gene mutation carriers in 57% of cases. Collectively, an abnormal MSI result, IHC result, or either an abnormal MSI or IHC result correlated with the presence of a germline mutation in 58% (23/40), 70% (28/40), and 73% (29/40) of cases, respectively. This correlation

Table 5. Correlation of MSI Status with IHC in Lynch Syndrome-Associated Adenomas

	MSI-H/MSI-L (n = 23)	MSS (n = 17)	P value
No loss	1	11	
MLH1 loss	15	6	
MSH2 loss	7	0	<0.001

between microsatellite status and expression of MMR proteins is shown in Table 5.

MSI and IHC Testing in Hyperplastic Polyps

In our cohort of Lynch syndrome patients undergoing surveillance colonoscopy, five hyperplastic polyps were identified. MSI and IHC analyses were performed on these polyps (Table 6). Three polyps from the same patient that carried an *MLH1* germline mutation were mixed polyps showing components of both hyperplastic polyp and tubular adenoma. The median size of these three polyps was 11.3 mm and all were located proximal to the splenic flexure. Microdissection was performed to separate these two components, and the results of the adenomatous components of these polyps were described above. In these three mixed polyps, the hyperplastic epithelium revealed preserved MLH1 staining and no MSI was observed in the two polyps in which MSI testing was performed. Two pure hyperplastic polyps were also removed from two carriers of an *MSH2* germline mutation. The median size of these polyps was 18.5 mm; one was located in the sigmoid and the other in the transverse colon. Staining for MSH2 by immunostaining was preserved in both cases, and both were MSS. Representative IHC images are shown in Figure 3B.

Discussion

The goal of this study was to determine the frequency of microsatellite instability and loss of immunostaining for MMR proteins in pre-cancerous adenomas of genetically defined Lynch syndrome patients. As the recognition of Lynch syndrome and its associated risk of other extra-colonic malignancies increases, a larger number of at-risk and pre-symptomatic individuals are presenting for genetic counseling. In such cases, adenomas may be the only tissue available, and analysis of these polyps for deficient mismatch repair may be a suitable alternative to testing of colon cancer samples. It is important to note that microsatellite instability and loss of MMR proteins, as shown by IHC analysis, are uncommon among adenomas in the general population. In a large series of 378 patients with adenomatous polyps, only six (1.6%) had at least one adenoma with MSI, and five of these six patients were indeed carriers of a germline MMR gene mutation.²⁶ We have demonstrated that a combined approach of MSI and IHC testing in adenomas is 73% sensitive in the detection of Lynch syndrome.

The particular strength of this study is that it represents the largest analysis of adenomas from patients with

Table 6. Clinicopathological Findings in Mixed and Hyperplastic Polyps

Case	Sex/Age (years)	Size (mm)	Histology	Adenoma location	Mutated gene	Type of mutation	IHC results	MSI status
1	F/52	20	TA/HP	P	<i>MLH1</i>	H718P	Loss/Preserved	MSS
	57	4	TA/HP	P			Loss/Preserved	ND
	58	10	TA/HP	P			Preserved	MSS
2	F/68	24	HP	D	<i>MSH2</i>	2116delG	Preserved	MSS
3	M/48	13	HP	P	<i>MSH2</i>	E48X	Preserved	MSS

M, male; F, female; TA, tubular adenoma; HP, hyperplastic polyp; D, distal; P, proximal; IHC, immunohistochemistry; MSI, microsatellite instability; MSS, microsatellite stable; ND, not done.

Lynch syndrome who have been unambiguously defined by a germline mutation. Prior reports have sought to address the role of MSI and IHC testing in adenomas, but have defined Lynch syndrome more broadly (fulfillment of either genetic or clinical [Amsterdam] criteria). Unfortunately, the correlation between the Amsterdam criteria and a germline mutation is imperfect, and many families who fulfill the Amsterdam criteria may indeed carry an alternate diagnosis, such as Syndrome X.²⁷ A germline mutation thus serves as the most precise way to diagnose Lynch syndrome. Our analysis was restricted to polyps from individuals who carried a defined mismatch repair gene mutation and therefore represents the largest group of true Lynch syndrome patients analyzed.

Iino et al examined 30 adenomas from 24 patients with Lynch syndrome.²⁸ However, only 10 of these patients had a defined germline mutation in *MLH1* or *MSH2*. Fifty-three percent of all adenomas displayed high levels of MSI. Among the 15 adenomas from the 10 patients with a known germline mutation in *MLH1* or *MSH2*, loss of MLH1 or MSH2 protein by IHC analysis was seen in all cases. DeJong analyzed 31 adenomas from 22 carriers of a germline *MLH1* or *MSH2* mutation, and loss of staining by IHC testing was observed in 65% of adenomas.²⁹ No MSI analysis was performed. Finally, Halvarsson included 32 adenomas from 23 patients with a known germline mutation in *MLH1* or *MSH2*, and an abnormal IHC result was observed in 66% of polyps.³⁰ No analysis of *MSH6* was performed in any of these studies. If adenomas from patients with an *MSH6* mutation are excluded from our analysis, then the sensitivity of a combined approach with MSI and IHC analyses rises to 78% in our study.

Our findings suggest that mutations in *MSH6* are less likely to produce abnormal MSI or IHC results in colorectal adenomas. This is consistent with the attenuated phenotype sometimes observed in *MSH6* kindreds and the greater likelihood that cancers in patients with germline *MSH6* mutations display an MSI-L as opposed to an MSI-H phenotype.³¹ Two of these proximally located *MSH6* adenomas developed synchronously greater than 5 cm away from a colorectal cancer located in the transverse colon, and absence of *MSH6* nuclear staining and MSI was observed in this cancer. Although some have observed similar patterns between synchronous adenomas and cancers in Lynch syndrome, our results are consistent with a previous study suggesting that Lynch syndrome-associated adenomas developing at a distance of more than 5 cm away from a carcinoma do not show MSI and express all MMR proteins.^{32,33} We cannot

rule out the possibility that these particular adenomas were "sporadic" and did not arise in conjunction with the underlying *MSH6* mutation. In agreement with previous observations, our data seem to suggest that the MMR phenotype may not be necessary for adenoma formation in patients with Lynch syndrome.^{25,33–35} Although some adenomas in these patients may arise as a consequence of dysfunctional DNA mismatch repair, others may be initiated as sporadic tumors that do not display microsatellite instability. Some colorectal cancers in carriers of an MMR gene mutation may indeed exhibit preserved IHC staining and MSS.³⁶

The combination of histological and molecular analyses is a powerful tool in the assessment of MMR, even in pre-cancerous lesions. Interestingly, the concordance between MSI and IHC testing in our cohort was good but not perfect. IHC analysis was more sensitive than MSI analysis. Six adenomas, all from carriers of an *MLH1* germline mutation, exhibited loss of protein expression, but no evidence of microsatellite instability. This finding suggests a MMR protein deficiency that has not yet manifested microsatellite instability. It is not inconceivable that loss of MMR protein may occur before the development of MSI, which may require multiple rounds of cell division before the appearance of this phenotype. Alternatively, these results may be due to contamination of DNA from normal stromal or inflammatory cells. Relying on IHC analysis alone would have missed one case, a 6-mm polyp located in the left colon from a carrier of an *MLH1* gene mutation. Since multiple polyps were analyzed from this patient, a possible explanation for the preserved IHC staining may be differences in the second mutational hit, leading in some cases to detection of a non-functional or truncated MMR protein. From a technical perspective, these discordant results could also be explained by the undefined binding site of the commonly used MLH1 antibody (a murine IgG monoclonal antibody raised against full-length MLH1 protein).^{37–39}

We found the highest rates of MSI and loss of immunostaining in large (≥ 5 mm) and high-grade dysplastic adenomas. Microsatellite instability and IHC loss of staining was detected in 65% and 79% of the larger adenomas, respectively. Only the latter relationship between loss of staining by IHC and polyp size was statistically significant. In our series, all high-grade dysplastic adenomas showed MSI ($P = 0.006$) in association with loss of staining ($P = 0.04$). Despite the right-sided predominance of our Lynch-associated adenomas, we surprisingly detected MSI and loss of MMR protein staining

more frequently in distally located adenomas. All of the distal adenomas showed MSI compared with 41% (12/29) of the proximal ($P = 0.0008$) polyps. Loss of staining was also reported in 85% (11/13) of the adenomas distal to the splenic flexure. These findings suggest that despite the right-sided predilection for Lynch-associated cancers, distally located adenomas should be considered to have equal malignant potential.

IHC and MSI testing correctly identified *MLH1* gene mutation carriers in 100% and 67% of cases, respectively, whereas in *MSH2* gene mutation carriers, loss of protein immunostaining and microsatellite instability were detected in 57% of cases. No abnormalities in IHC and MSI testing were observed in adenomas from the two patients with an *MSH6* gene mutation. Overall, it appears that carriers of an *MSH2* or *MSH6* germline mutation are less likely to show loss of staining and/or microsatellite instability; only four of the nine carriers exhibited abnormal MSI and IHC results whereas all carriers of an *MLH1* germline mutation exhibited abnormal MSI and IHC results. However, it should be noted that all adenomas with preserved staining and stability of the microsatellites were small (median size 4 mm) and did not exhibit high-grade dysplasia.

Our series also suggests that carriers of an *MLH1* germline mutation may develop more adenomas than carriers of an *MSH2* or *MSH6* mutation. However, this is likely a reflection of our smaller cohort, as a recent analysis of 695 patients with a proven germline MMR mutation did not identify significant differences in the prevalence of adenomas among individuals with *MLH1*, *MSH2*, or *MSH6* mutations.⁴⁰

An area of particular interest is whether MMR defects have a role in the development of hyperplastic and serrated polyps and whether these polyps indeed have malignant potential. Traditionally, hyperplastic polyps have been regarded as benign lesions with no potential for neoplastic progression. However, the recent findings of mutations in *BRAF* as well as microsatellite instability in certain hyperplastic/serrated polyps suggest that this paradigm may need re-evaluation and that these lesions may indeed be precursors of sporadic MSI-H colorectal cancers.^{41–47} It is unknown whether hyperplastic polyps that arise in the context of Lynch syndrome may also be pre-cancerous. In the present study we analyzed five hyperplastic polyps and found stability of the microsatellites as well as preserved immunostaining in all cases. Interestingly, several of the polyps analyzed were mixed adenomatous/hyperplastic polyps, and abnormal MSI and IHC results were confined to the adenomatous compartment. These results, although limited by the sample size, suggest that it is unlikely that hyperplastic polyps play a significant role in the pathogenesis of microsatellite unstable tumors in subjects with a germline MMR gene mutation.^{48,49} None of the polyps in the present study was classified as a serrated adenoma.

In conclusion, molecular analysis of colorectal adenomas may have a role in the workup of suspected Lynch syndrome. The combination of both MSI analysis and IHC staining for MMR proteins detected DNA repair deficiency in 73% of the Lynch-associated adenomas, and

this included adenomas smaller than 5 mm. Thus, in the workup of patients suspected to have a germline MMR gene mutation, it is reasonable to begin with MSI and IHC analyses of adenomas, and our data suggest that IHC testing alone is nearly as sensitive as a combined approach. Adenoma size does not appear to be consistently correlated with a positive test result, so small adenomas should not be excluded from analysis. Positive results can be used to direct germline genetic testing. However, a negative MSI or IHC test result in an adenoma should be interpreted cautiously and cannot be used to formally exclude the diagnosis of Lynch syndrome if other clinical features suggest the diagnosis. This is particularly true for *MSH6* mutation carriers. Nevertheless, this approach would expand the diagnostic testing options in cases with suspected Lynch syndrome and increase the opportunities to recognize the syndrome before the development of invasive cancer.

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