Genetic and Epigenetic Modification of *MLH1* Accounts for a Major Share of Microsatellite-Unstable Colorectal Cancers

Shannon A. Kuusmanen,* Mari T. Holmberg,*
Reijo Salovaara,*† Albert de la Chapelle,‡ and
Päivi Peltomäki‡

From the Departments of Medical Genetics* and Pathology,†
Haartman Institute, University of Helsinki, Helsinki, Finland;
and the Division of Human Cancer Genetics,‡ Comprehensive
Cancer Center, The Ohio State University, Columbus, Ohio

Microsatellite instability (MSI) is a hallmark of hereditary nonpolyposis colorectal cancer, and in these patients, results from inherited defects in DNA mismatch repair genes, mostly *MSH2* and *MLH1*. MSI also occurs in 15% of sporadic colorectal cancers, but in these tumors, its basis is less well characterized. We investigated 46 sporadic MSI+ colorectal cancers for changes in *MSH2* and *MLH1* protein expression, followed by the analysis of somatic mutation, loss of heterozygosity (LOH), and promoter hypermethylation as possible underlying defects. Most cases (36/46, 78%) showed lost or reduced *MLH1* expression. Among these, a majority (83%) was associated with *MLH1* promoter hypermethylation, whereas the rates of LOH and somatic mutation of *MLH1* were 24% and 13%, respectively. Hypermethylation and LOH were inversely correlated, suggesting that they had alternative functions in the inactivation of *MLH1*. *MSH2* expression was lost in 7/46 (15%), and of these, 2 (29%) showed LOH and/or somatic mutation of *MSH2*. We conclude that most sporadic MSI+ colorectal cancers have an *MLH1*-associated etiology and that epigenetic modification is a major mechanism of *MLH1* inactivation. Moreover, we found a significantly lower prevalence for *MLH1* promoter hypermethylation in hereditary nonpolyposis colorectal cancer tumors with *MLH1* germline mutations (12/26, 46%), which might explain some differences that are known to occur in the clinicopathological characteristics and tumorigenic pathways between sporadic and hereditary MSI+ colorectal cancers. (*Am J Pathol* 2000, 156:1773–1779)

In hereditary nonpolyposis colorectal cancer (HNPCC) tumors, a two-hit mechanism for DNA mismatch repair gene inactivation has been postulated to underlie microsatellite instability (MSI).^{1–3} A germline mutation may be

accompanied by the loss and/or somatic mutation of the wild-type allele,^{2,4,5} yet a considerable proportion (up to 65% of tumors in the studies cited above) shows no identifiable second hit. Until recently, the basis of MSI has been even less clear in sporadic MSI+ cases that display lower frequencies of loss of heterozygosity (LOH) and somatic mutation of *MSH2* and *MLH1* as compared to colorectal cancers from HNPCC patients.^{2,4,6} The discovery of frequent hypermethylation at the *MLH1* promoter region has offered an alternative explanation for the inactivation of this gene in sporadic MSI+ tumors.^{7–10} By contrast, the *MSH2* gene promoter has not been found to show hypermethylation.^{8,9}

Many previous studies have typically been restricted to only one or two possible mechanisms of DNA mismatch repair gene inactivation at a time and have used unselected series representing a mixture of hereditary and sporadic tumors. These drawbacks have made it difficult to obtain a comprehensive overview of the relative contributions of different mechanisms leading to MSI. The present investigation focused on 46 sporadic MSI+ colorectal cancers in which *MSH2* and *MLH1* germline mutations had been excluded by direct sequencing. A sequential approach was applied to investigate these tumors for *MSH2* and *MLH1* alterations including loss of protein expression, somatic mutation, LOH, and promoter hypermethylation. A cohort of MSI+ colorectal tumors from HNPCC patients was studied for comparison. We demonstrate that the *MSH2* versus *MLH1* genes as well as sporadic versus hereditary cases are differently involved in the inactivation by these different mechanisms, providing important insights into the basis of MSI in colorectal cancer.

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S. A. K. and M. T. H. contributed equally to this work.

Address reprint requests to Dr. Päivi Peltomäki, Division of Human Cancer Genetics, Comprehensive Cancer Center, The Ohio State University, 690 Medical Research Facility, 420 W. 12th Avenue, Columbus, OH 43210. E-mail: peltomaki-1@medctr.osu.edu.

Materials and Methods

Patients and Samples

Paired fresh-frozen or paraffin-derived normal colonic mucosa and colorectal tumor samples from sporadic cases were investigated, representing a collection of 509 consecutive tumors previously analyzed for MSI.¹¹ Among these were tumors that had shown high-degree MSI (MSI-H) but no germline mutations of the *MSH2* or *MLH1* genes by direct sequencing ($n = 51$). In addition, tumor and normal tissue pairs available from HNPCC patients with inherited mutations in DNA mismatch repair genes (*MLH1* mutation in 26 cases and *MSH2* mutation in one case^{6,12,13}) were investigated. All patients gave informed consent before sample collection, in accordance with institutional guidelines.

Immunohistochemical Analysis

Four-micrometer sections from paraffin blocks were mounted on 3-aminopropyl-triethoxy-silane (Sigma, St. Louis, MO) coated slides and dried at 37°C. The sections were deparaffinized in xylene and rehydrated through a graded alcohol series to distilled water. Antigen retrieval was performed in 0.01-mol/L citrate buffer heated in a microwave oven at high power four times for 5 minutes each. The samples were then cooled to room temperature and washed in phosphate-buffered saline. For immunohistochemical analysis, avidin-biotin-conjugated immunoperoxidase technique was applied, using a commercial Elite ABC kit (Vectastain, Vector Laboratories, Burlingame, CA). Endogenous peroxidase activity was blocked by the incubation of the slides in hydrogen peroxide and methanol. Nonspecific antibody binding was quenched by incubation of the sections with nonimmune horse serum. Primary antibody was incubated with the sections overnight, followed by incubation in biotinylated secondary antibody and peroxidase-labeled avidin-biotin complex for 30 minutes. All antibody dilutions were in phosphate-buffered saline, pH 7.2, and incubations were carried out in humid chambers at room temperature. The following monoclonal primary antibodies were used. For *MLH1*, we used clone G168-728 from PharMingen (San Diego, CA), raised against full-length human MLH1 protein. For *MSH2*, we used clone G219-1129 from PharMingen, raised against full-length human MSH2 protein, as well as clone FE 11 from Oncogene Sciences (Uniondale, NY), raised against the COOH-terminal fragment of human MSH2 protein. Staining results were visualized by incubating the sections in 3-amino-9-ethylcarbazole solution (Sigma) for 15 minutes at room temperature. Sections were counterstained in Mayer's hematoxylin, rinsed in water, and mounted in aqueous media (Aqua-mount, BDH, Poole, UK) for microscopic evaluation and photography.

Methylation Analysis

A polymerase chain reaction-based assay was applied that relies on the inability of the *HpaII* restriction enzyme

to cut CCGG sequences with the internal cytosine methylated. Four *HpaII* sites contained at the *MLH1* promoter region were studied using the primers and conditions described in Kuismanen et al.¹⁰

LOH Analysis

Three microsatellite markers from the *MLH1* region were used that showed the highest rates of deletions in a previous study.² Marker D3S1611 is located in intron 12 of *MLH1* (our unpublished data), whereas D3S1029 and D3S1283 flank the gene on either side at 5-cM distance. For *MSH2*, D2S391 was used, flanked by D2S2259 and D2S123, together encompassing a region of 9 cM around *MSH2* (<ftp://ftp.genethon.fr>). LOH at 1–3 loci was recorded as LOH, whereas constitutional homozygosity and/or MSI at all three loci were interpreted as an uninformative result. In some cases, additional LOH data were obtained by denaturant gradient gel electrophoresis, taking advantage of intragenic point mutations and sequence polymorphisms.

Mutation Analysis

All cases included in the present study had been screened for germline mutations of *MSH2* and *MLH1*.^{6,11–13} Thirty-one sporadic colorectal cancer samples and two HNPCC cases were screened for somatic mutations in *MSH2* and *MLH1* by two-dimensional DNA electrophoresis, as described in Wu et al.⁶

Statistical Analysis

Fisher's exact test (two-tailed) was used to test statistical significance for the observed differences between groups.

Results and Discussion

Basis of MSI in Sporadic Colorectal Tumors

Fifty-one MSI+ colorectal tumors that showed no germline mutation in the two major HNPCC-associated genes, *MSH2* and *MLH1*, were subjected to immunohistochemical analysis of the MSH2 and MLH1 proteins, and the results were fully interpretable in 46 cases (Table 1 and Figure 1). All but 9 (80%) showed the involvement of either *MLH1* or *MSH2* or both. The predominant involvement of *MLH1* was suggested by the fact that most cases (36/46, 78%) were associated with lost or reduced MLH1 expression, and only 7 (15%) showed decreased expression of MSH2. Our findings are compatible with those of Thibodeau et al,¹⁴ who found reduced expression of MLH1 in 91% of unselected colorectal carcinomas with the MSI-H phenotype. By contrast, Dietmaier et al¹⁵ reported a roughly equal involvement of *MSH2* and *MLH1* by immunohistochemical analysis of MSI-H tumors (8 and 6 tumors, respectively, showing reduced expression). This discrepancy could be due to the fact that the series

Table 1. Summary of Expression, LOH, Mutation, and DNA Methylation Data as well as Site of Origin in Sporadic MSI+ tumors

Case no.	Protein expression		LOH		Somatic mutation	MLH1 promoter hypermethylation	Tumor location
	MLH1	MSH2	3p/MLH1	2p/MSH2			
Decreased MLH1 expression, normal MSH2 expression							
17	*	**	N		None	+	P
18	(*)	***	LOH		None	+	P
39	*	**	N		None	+	P
43	(*)	**	N		MLH1 exon 11, c. 325, Arg>Gln	+	P
139	*	***	N		None	+	P
144	(*)	***	N		None	—	P
171	(*)	**	N		None	+	P
215	(*)	**	LOH		None	+	P
229	*	**	LOH		None	—	D
239	*	**	U		None	+	R
277	*	**	N			+	P
287	(*)	**	N			+	P
331	*	**	N			+	P
355	(*)	**	U			+	P
371	(*)	**	N			+	P
396	(*)	**	N			+	P
406	(*)	**	LOH			+	P
426	(*)	**	U			+	P
459	(*)	**	N			+	P
469	(*)	**	N			+	P
484	(*)	**	LOH			+	D
493	(*)	**	U			+	P
500	(*)	**	N			+	P
543	(*)	**	LOH			—	D
549	*	**	N			+	P
555	*	***	U			+	P
560	(*)	**	U			+	P
567	(*)	**	U			—	P
575	*	**	N			+	P
578	(*)	**	N			+	P
Decreased MLH1 and MSH2 expression							
10	(*)	(*)	N	N	MLH1 exon 2, c. 54, Gly>Glu	+	P
11	(*)	(*)	N	U	MSH2 exon 7, c. 406 Arg>stop	+	R
56	(*)	*	LOH	LOH	MSH2 exon 13, c. 693, del T	—	P
136	*	*	N	N	None	—	P
226	(*)	*	N	N	None	+	P
233	(*)	*	N	N	None	+	P
Decreased MSH2 expression, normal MLH1 expression							
220	**	(*)	LOH	N	None	—	P
Normal MLH1 and MSH2 expression							
184	***	***	LOH	LOH	None	—	R
411	**	**	N	N		+	P
440	***	**	N	N		+	P
449	***	**	N	N		—	P
450	**	***	U	N		+	P
451	**	**	U	N		+	D
521	**	**	N	U		+	P
532	**	**	N	N		—	R
568	**	**	U	U		+	P

Protein expression was graded as follows: (*), no visible staining; *, staining in <25%; **, staining in 25–49%; ***, staining in ≥50% of cancer cell nuclei. In the LOH column, N denotes no change, LOH denotes loss of constitutional heterozygosity, and U denotes uninformative. DNA methylation changes were recorded as the presence (+) or absence (—) of methylation at one or several of the four *HpaII* sites of the MLH1 promoter region in tumor tissue. Tumor location was indicated as P, proximal colon (ie, before the splenic flexure); D, distal colon (after the splenic flexure); R, rectum. In all columns, blank denotes not studied.

studied by Dietmaier et al¹⁵ was a combination of sporadic and hereditary cases, whereas our tumors were truly sporadic based on the absence of *MSH2* and *MLH1* germline mutations.

In the *MLH1*-linked group, at least one genetic or epigenetic defect that might explain the altered expression

was present in all cases but three (tumors no. 144, 567, and 136), ie, 33/36 (92%). First, MLH1 promoter hypermethylation occurred in 30 cases. The present technique does not allow us to distinguish whether hypermethylation affected both *MLH1* alleles or only one, but biallelic involvement is common on the basis of previous stud-

Figure 1. Graphic presentation of the concurrence of the *MLH1* and *MSH2* changes in a series of 46 MSI+ sporadic colorectal cancers. The number of cases showing each type of alteration is given within the bars.

ies,¹⁶ and, if that were true, hypermethylation alone could explain almost all cases of extinct *MLH1* expression. Functional studies have established that *MLH1* promoter hypermethylation can indeed effectively silence the *MLH1* gene.^{8,16} In a minority of cases (nos. 18, 43, 215, 406, 484, and 10), LOH and/or somatic mutation occurred together with *MLH1* promoter hypermethylation and could constitute one of the hits presumably required for biallelic inactivation.

In the *MSH2*-associated group, a somatic defect (loss or mutation of *MSH2*) was detected in 2/7 tumors (29%). In one case (tumor no. 56), biallelic inactivation of *MSH2* was suggested as there was a somatic mutation in one allele and loss of the other allele.⁶ The possibility of promoter hypermethylation underlying *MSH2* inactivation was not addressed in the present investigation, as two earlier studies^{8,9} had demonstrated that, unlike *MLH1*, the *MSH2* promoter is not prone to hypermethylation in MSI+ tumors. Although most MSI+ tumors were associated with a reduction of either *MLH1* or *MSH2* protein alone, the expression of both was decreased in six cases, and in half of these (tumors no. 10, 11, and 56), somatic hits affecting both *MSH2* and *MLH1* were detected.

Despite the MSI+ phenotype, the expression of *MLH1* and *MSH2* was unaltered in 9 cases. The expression of a mutated nonfunctional protein remains a possibility in this group, since only one tumor was available for mutation analysis (with no mutation present). Alternatively, defects in other genes, such as the DNA mismatch repair genes *MSH3* and *MSH6*¹⁷ or DNA polymerase delta¹⁸ may account for MSI in these tumors. As a whole, our present and previous¹⁰ data combined with reports by others⁷⁻⁹ suggest that in a vast majority of cases, promoter hypermethylation is associated with immunohistochemically and/or biochemically demonstrable inactivation of *MLH1* as a functional consequence. However, this is not invariably the case, as illustrated by the fact that hypermethylation was present in six tumors without any apparent

reduction in protein expression. It is possible that hypermethylation affected only one of the two *MLH1* alleles, as proposed,⁹ or despite affecting some CpG (in this case, *HpaII*) sites, left some other sites intact whose methylation might be necessary for the silencing of this gene.¹⁹ Finally, recent observations^{10,20} suggest that *MLH1* promoter methylation should be viewed as being part of a more widespread hypermethylation tendency that characterizes MSI+ colorectal tumors, but is rare in MSI- tumors. Indeed, the frequency of *MLH1* promoter hypermethylation without reduced protein expression (6/36, 17%) was comparable to the frequency of *MLH1* hypermethylation in MSI- tumors (11%), as determined previously.¹⁰ Thus, although typically clones with inactive *MLH1* and defective DNA mismatch repair after *MLH1* promoter hypermethylation would be selected for during tumorigenesis, hypermethylation might occasionally have other targets, and the occurrence of *MLH1* promoter hypermethylation in the same cells could be merely coincidental and without functional consequences.

Somatic Events in Hereditary MSI+ Colorectal Cancers

To investigate whether the presence of a germline mutation as an inherited defect in every cell had an effect on the nature of the somatic lesion, we investigated colorectal tumors from 27 HNPCC patients for LOH and promoter hypermethylation (Table 2). All cases were included in which the predisposing mutation was known and samples were available. With one exception, all were *MLH1*-linked, reflecting the fact that *MLH1* germline mutations predominate in the studied population, for reasons that are not fully understood.¹³ Among 26 *MLH1*-linked HNPCC tumors, promoter hypermethylation was significantly less prevalent than in the sporadic MSI+ tumors showing reduced expression of *MLH1* (12/26, 46% vs. 30/36, 83%, $P = 0.003$). By contrast, the LOH rates were

Table 2. Summary of LOH, DNA Methylation, Mutation Data, and Site of Origin in HNPCC MSI+ tumors

Case no.	LOH		MLH1 promoter hypermethylation	Germline mutation	Tumor location
	MSH2	MLH1			
MLH1-linked					
6		N	+	MLH1, genomic deletion of exons 3–5	P
34		N	+	MLH1, exon 4, c. 107, Ile>Arg	P
56		N	+	MLH1, exon 4, c. 107, Ile>Arg	P
5		U	–	MLH1, splice acceptor of exon 6, g>a at 454-1	P
58		N	+	MLH1, splice acceptor of exon 6, g>a at 454-1	P
24		N	+	MLH1, splice acceptor of exon 12, g>a at 1039-1	D
25		U	–	MLH1, splice acceptor of exon 12, g–>a at 1039-1	P
26		LOH	–	MLH1, splice acceptor of exon 14, g>t at 1559-1	P
2		N	+	MLH1, exon 16, 3.5-kb genomic deletion	P
13		LOH	–	MLH1, exon 16, 3.5-kb genomic deletion	R
14		U	+	MLH1, exon 16, 3.5-kb genomic deletion	P
324		N	+	MLH1, exon 16, 3.5-kb genomic deletion	R
17		LOH	–	MLH1, exon 16, 3.5-kb genomic deletion	P
430		U	–	MLH1, exon 16, 3.5-kb genomic deletion	P
38		N	–	MLH1, exon 16, 3.5-kb genomic deletion	R
54		U	+	MLH1, exon 16, 3.5-kb genomic deletion	P
1		N	+	MLH1, exon 16, 3.5-kb genomic deletion	P
3		N	–	MLH1, exon 16, 3.5-kb genomic deletion	P
4		LOH	–	MLH1, exon 16, 3.5-kb genomic deletion	R
10		LOH	–	MLH1, exon 16, 3.5-kb genomic deletion	R
12		N	–	MLH1, exon 16, 3.5-kb genomic deletion	NK
28		N	+	MLH1, exon 16, 3.5-kb genomic deletion	D
45		N	–	MLH1, exon 17, c. 659, Arg>stop	D
52		LOH	–	MLH1, exon 17, c. 659, Arg>stop	P
7		LOH	+	MLH1, exon 17, c. 659, Arg>Pro	P
8		N	–	MLH1, exon 17, c. 659, Arg>Pro	R
MSH2-linked					
57	LOH	N	–	MSH2, splice acceptor of exon 9, g>t at 1387-1	D/R

The MLH1-linked cases are grouped according to the site of the germline mutation in this gene. For the abbreviations and interpretation of the results, see notes to Table 1.

NK, not known.

comparable (7/21, 33% in HNPCC vs. 7/29, 24% in sporadic MSI+ tumors). Combining hereditary and sporadic MSI+ tumors, hypermethylation and LOH were mutually exclusive (LOH was present in 5/34, 15% vs. 9/16, 56% among informative cases with *versus* without hypermethylation, $P = 0.005$), suggesting that they had similar functions in *MLH1* inactivation. In particular, with just one exception, HNPCC tumors carrying the inherited mutation as the first hit did not show promoter hypermethylation at all, if LOH was present (Table 2), suggesting that the

wild-type allele rather than the mutated allele was the preferential target for hypermethylation, the putative second hit. In the single *MSH2*-linked case, the germline mutation was accompanied by LOH as an acquired event.

Pathogenetic Implications

MLH1 promoter hypermethylation was a feature of proximal tumors in the sporadic MSI+ group, whereas the

Table 3. Correlation of MLH1 Promoter Methylation and LOH Status *versus* Tumor Location in the Bowel

	Number of cases			
	Sporadic		HNPCC (<i>MLH1</i> -linked)	
	Proximal	Distal	Proximal	Distal
Hypermethylation present	32/36 (89%)	4/36 (11%)	9/12 (75%)	3/12 (25%)
Hypermethylation absent	6/10 (60%)	4/10 (40%)	7/13 (54%)	6/13 (46%)
	$P = 0.05$		NS	
LOH present	4/7 (57%)	3/7 (43%)	4/7 (57%)	3/7 (43%)
LOH absent*	20/21 (95%)	1/21 (5%)	7/13 (54%)	6/13 (46%)
	$P = 0.04$		NS	

Sites before the splenic flexure were considered proximal, those after the splenic flexure, including the rectum, were considered distal. Values of statistical significance are also given (NS, nonsignificant).

*Based on informative cases.

HNPCC group showed no statistically significant association between DNA methylation status and tumor location (Table 3). If, as suggested,^{10,20} MLH1 promoter hypermethylation represents a more generalized phenomenon that is associated with tumor formation preferentially in the proximal colon, and if such hypermethylation is significantly more common in sporadic than in hereditary MSI+ tumors (this study), this might explain the relatively higher percentage of proximal tumors in the former group (83 to 94% vs. 70%, respectively²¹). The different prevalence for hypermethylation combined with its possible regulatory consequences might thus be reflected in pathogenetic differences between the two groups of tumors despite a similar MSI+ phenotype. An example of a gene whose regulation by hypermethylation could make a difference is APC, an important gatekeeper of colon tumorigenesis²²; in fact, this gene was recently found to show promoter hypermethylation specifically in proximal tumors.²³ LOH in the MLH1 region was associated with distal sporadic MSI+ tumors (Table 3), which is in agreement with the inverse correlation between LOH and MLH1 promoter hypermethylation, as noted previously. By contrast, the LOH status showed no correlation with tumor location among HNPCC tumors. It is possible that LOH in general is important in the development of distal sporadic tumors,²⁴ no matter whether MSI+ or MSI-, whereas in HNPCC, the inherited DNA mismatch repair deficiency *per se* is probably a strong independent determinant of tumor location.

In summary, we have shown that most sporadic MSI+ tumors have an MLH1-associated etiology and that epigenetic modification is a major mechanism of MLH1 inactivation. Further studies should clarify the cause and mechanisms of such hypermethylation as well as its tumorigenic targets besides the MLH1 gene. In addition, further work is necessary to identify the basis of MSI in the minority (20%) of sporadic MSI+ colorectal cancers not associated with decreased expression of the MSH2 or MLH1 proteins.

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