

# Characterization of Sporadic Colon Cancer by Patterns of Genomic Instability<sup>1</sup>

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## ABSTRACT

Colorectal cancer (CRC) can progress through two pathways of genomic instability: chromosomal (CIN) and microsatellite instability (MSI). We hypothesized that these two pathways are not always independent and that some tumors therefore show a significant degree of overlap between these two mechanisms. We classified 209 high-risk stage II and stage III sporadic CRCs based on their MSI status, using a National Cancer Institute-recommended panel of microsatellite markers, and also identified MSI-associated mutations of CRC target genes such as *TGFBR1I*. Evidence for CIN was gathered by identifying loss of heterozygosity (LOH) events on chromosomal arms 1p, 2p, 3p, 5q, 17p, and 18q, which are regions harboring mismatch-repair and tumor-suppressor genes that are significant in CRC development. Results of all molecular markers tested were correlated with clinicopathological variables of the cohort, including treatment outcome. Of the 209 cases, 65% cancers were microsatellite stable, 21% were MSI-low, and 14% were MSI-high (MSI-H). Overall, 51% of the tumors had at least one LOH event, with most frequent chromosomal losses observed on 18q (72.5%), followed by 5q (22%), 17p (21%), and 3p (14%). Interestingly, we observed a significant degree of overlap between MSI and CIN pathways. Of 107 cancers with LOH events, 7 (6.5%) were also MSI-H, and of 30 cancers that were MSI-H, 7 (23.3%) also had one or more LOH events. We also found that 37.8% of microsatellite-stable cancers had no LOH events identified, thus comprising a subgroup of tumors that were not representative of either of these two pathways of genomic instability. Our data suggest that molecular mechanisms of genomic instability are not necessarily independent and may not be fully defined by either the MSI or CIN pathways.

## INTRODUCTION

Colorectal carcinogenesis is characterized by the successive accumulation of mutations in genes controlling epithelial cell growth and differentiation. The term “genomic instability” describes conditions whereby widespread loss of DNA integrity is perpetuated. The development of genomic instability is an important event in the multistep progression of colorectal carcinogenesis. Two apparently independent pathways of genomic instability have been identified (1, 2). The first, and more common, pathway is characterized by the sequential inac-

tivation of tumor-suppressor genes, such as *APC*<sup>6</sup> (chromosome 5q), *p53* (chromosome 17p), and *DCC*, *SMAD2*, and *SMAD4* (chromosome 18q). Tumors generated through this “suppressor” pathway display CIN with frequent cytogenetic abnormalities and allelic losses (3, 4). The precise mechanism driving the process of chromosomal instability is not well understood. A second pathway is characteristic of tumors from patients with HNPCC, an autosomal-dominant condition that accounts for 2–3% of all colorectal carcinomas. The hallmark of this alternative “mutator” pathway is widespread MSI, which is characterized by the accumulation of somatic alterations in the length of simple, repeated nucleotide sequences called “microsatellites.” The MSI-H found in tumors from patients with HNPCC results from defects in the DNA MMR system (5, 6) that are caused by germ-line mutations of the MMR genes, the most common being *hMLH1* or *hMSH2* (7, 8).

MSI-H has also been identified in ~10% of sporadic colon carcinomas. In these cases, mutations of *hMLH1* and *hMSH2* are rarely found (9, 10). Recent studies indicate that *hMLH1* inactivation by promoter hypermethylation also produces the MSI-H phenotype in sporadic colorectal cancers and is responsible for most, if not all of the sporadic colorectal cancers with MSI-H (11, 12). MSI-H colorectal cancers do not exhibit gross cytogenetic abnormalities; they display allelic losses at tumor suppressor loci infrequently, and they are not generally aneuploid (13–16). Instead, these tumors accumulate slippage-induced frameshift mutations at microsatellite sequences. Some of these mutations occur in coding regions of specific genes that are implicated in tumor progression, such as *TGFBR1I*, *IGF1IR*, *hMSH3*, *hMSH6*, and *BAX* (17–20).

Colorectal carcinomas originating by the suppressor and the mutator pathways differ in several pathological features. Tumors with MSI-H, both sporadic and HNPCC-associated, may be more likely to arise in the proximal colon, demonstrate poor differentiation, have mucinous or medullary features, and display more prominent lymphocytic infiltration than seen in MSS tumors and MSI-L tumors (7, 13, 14, 21–23). Furthermore, patients with MSI-H tumors have a more favorable survival than do patients with MSI-L/MSS colorectal carcinomas (13, 14, 24, 25).

Although these two mechanisms of genomic instability can be distinguished from one another by their molecular characteristics, evidence suggests that there might be some degree of overlap. For example, it has been reported that LOH is occasionally a mechanism by which the wild-type allele of *hMLH1* is inactivated in some MSI-H tumors (26). It is also possible that colorectal cancers are initiated by mechanisms not involving persistent MSI or CIN. For example, emerging evidence indicates that epigenetic modification by the hypermethylation of the promoter regions of key tumor suppressor genes

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<sup>6</sup> The abbreviations used are: APC, adenomatous polyposis coli; CIN, chromosomal instability; HNPCC, hereditary nonpolyposis colorectal cancer; MSI, microsatellite instability; MSI-H, high-frequency MSI; MMR, mismatch repair; *TGFBR1I*, transforming growth factor  $\beta$  type II receptor; *IGF1IR*, insulin-like growth factor II receptor; MSS, microsatellite stable; MSI-L, low-frequency MSI; LOH, loss of heterozygosity; CALGB, Cancer and Leukemia Group B; UCSD, University of California at San Diego.

may play a critical role in the evolution and progression of many colorectal tumors (27). These data suggest that tumors arising because of promoter methylation may not progress independently through either the MSI or the CIN pathway. These findings further suggest that MSI and CIN might not represent totally distinct mechanisms and that multiple mechanisms might exist in some tumors if the combinations could provide additional growth advantages.

To date, no systematic study has determined the extent of overlap between the MSI and CIN pathways. In addition, available data do not fully address the question of whether every colorectal cancer bears genetic alterations related to one of these two well-understood mechanisms of genomic instability. The current study was therefore pursued to classify a large cohort of sporadic colorectal cancers based on their MSI and CIN status. We hypothesized that there would be some tumors showing a significant degree of overlap between these two mechanisms and that there might be some proportion of tumors that might not show evidence for involvement of either of these mutational pathways.

## MATERIALS AND METHODS

**Patients.** Clinical data and tumor specimens were obtained from a total of 209 patients with sporadic colorectal cancer treated by physicians associated with either CALGB or the UCSD. Institutional Review Board approval was granted for this study. Patients included in this study were considered to have sporadic tumors because their clinical presentation and family history did not suggest a diagnosis of either familial adenomatous polyposis (1) or HNPCC [as defined by the Amsterdam criteria (28)].

The CALGB cohort consisted of 168 individuals who received chemotherapy for colon cancer as part of CALGB Protocol 8896 (Intergroup 0089). These patients underwent surgical resection of an adenocarcinoma of the colon and were determined to have a high risk of tumor recurrence based on regional nodal disease (127 patients, stage III) or local extension of tumor with obstruction or perforation because of tumor (41 patients, stage II). All of the CALGB patients received adjuvant chemotherapy per Protocol 0089, which was a four-arm randomization of different 5-fluorouracil-based regimens that failed to show any outcome difference among the four different treatments. For this reason, outcome data used for the analyses described in this report were pooled for all patients in the CALGB cohort. Median clinical follow-up for the CALGB cohort, by the Kaplan-Meier method, was 8.25 years.

The UCSD cohort contained 41 individuals treated for colon cancer between January 1983 and November 1993. Of these 41 patients, 8 (19.5%) were stage II and 33 (80.5%) were stage III. Adjuvant chemotherapy was administered to 24.4% of the UCSD patients, with one stage II (12.5%) and nine stage III (27.3%) patients receiving this treatment. The median clinical follow-up for the UCSD patients is 6.5 years. Available median clinical follow-up for the combined patient population is 8.2 years.

The study cohort included patients from all regions of the United States and contained both patients who were treated at major academic medical centers and those cared for in community hospitals and clinics. The median age of the study population was 63.0 years, and 84.2% of patients were age 50 or older (Table 1). There was a higher percentage of male patients with stage II disease ( $P = 0.01$ ). Overall, 83.7% of the study patients were of Caucasian descent, with other races accounting for 16.3% of the total. The clinical characteristics of the CALGB and UCSD cohorts differed significantly in a few areas. Whereas the CALGB cohort was of uniform risk for recurrent disease (*i.e.*, node-positive or exhibiting tumor-associated obstruction or perforation), the UCSD cohort contained stage II patients who were not of the latter high-risk category. The CALGB patients were significantly younger (63.2 *versus* 68.8 years;  $P = 0.0004$ ), more likely to have received chemotherapy (100% *versus* 24.4%;  $P < 0.0001$ ), and less likely to be of Caucasian descent (88.6% *versus* 63.4%;  $P < 0.0001$ ). Other variables, including sex, tumor differentiation, tumor location, and tumor histological subtype, were the same for the two cohorts. Consistent with the clinical and pathological differences between the two cohorts, the UCSD patients had a significantly worse overall survival (log rank,  $P = 0.004$ ), although death from disease was not different between the two groups (log rank,  $P = 0.18$ ).

Table 1 Study cohort characteristics by stage

	Stages II & III		Stage II		Stage III		<i>P</i>
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	
Stage	209		49	23.4	160	76.6	
CALGB	168	80.4	41	83.7	127	79.4	
UCSD	41	19.6	8	16.3	33	20.6	
Age (yrs)	209		49		160		0.75
<50	33	15.8	9	18.4	24	15.0	
50-65	86	41.1	21	42.9	65	40.6	
>65	90	43.1	19	38.8	71	44.4	
Gender	209		49		160		0.01
Male	117	56.0	35	71.4	82	51.3	
Female	92	44.0	14	28.6	78	48.8	
Race	208		49		159		0.66
White	174	83.7	40	81.6	134	84.3	
Other	34	16.3	9	18.4	25	15.7	
Differentiation	202		41		155		0.03
Well	26	12.9	11	23.4	15	9.7	
Moderate	137	67.8	30	63.8	107	69.0	
Poor	39	19.3	6	12.8	33	21.3	
Lymph nodes	205		47		158		<0.0001
0	49	23.9	47	100.0	2	1.3	
1-3	109	53.2	0	0.0	109	69.0	
>4	47	22.9	0	0.0	47	29.7	
Tumor site	205		48		157		0.40
Proximal	96	46.8	25	52.1	71	45.2	
Distal	109	53.2	23	47.9	86	54.8	

<sup>a</sup> Four patients had multiple tumor sites and were excluded from the analysis.

**Analysis of Tumor Specimens.** Formalin-fixed, paraffin-embedded tissue specimens obtained at the time of surgery for primary treatment of colon cancer were collected from all study patients. At least one tissue block representative of tumor, and one separate block containing either normal colonic mucosa or a noninvolved lymph node were obtained for each patient. H&E-stained slides were prepared from three levels in each tissue block and examined by two independent reviewers (L. W. and C. C.) to assure the quality of material obtained for DNA analyses. One central review pathologist (C. C.) also evaluated the histological grade of all tumors (well, moderately, or poorly differentiated). Features evaluated from the accompanying pathology report and the prepared slides (if possible) included (*a*) anatomical site, (*b*) degree of local spread (pT), and (*c*) regional lymph node status (pN).

A clinical database for all study patients was maintained at the CALGB Statistical Center. Histological grading and laboratory analysis of the tumor specimens were performed without the knowledge of the patients' clinical data.

**Microdissection and DNA Amplification.** Serial sections from paraffin-embedded matched normal and neoplastic primary tissues (5  $\mu$ m) were stained with H&E, and representative normal and tumor regions were identified by microscopic examination. This reference slide was used to microdissect 100-150 cells, using a sterile scalpel blade under a dissecting microscope. Every effort was made to minimize contamination of tumor DNA with the normal mucosa and *vice versa*. Normal control tissue (nontumor) was obtained from histologically normal mucosa and/or normal lymph nodes. Genomic DNA was isolated from the paraffin-embedded microdomains removed from the slides using GeneReleaser (Bioventures Inc, Murfreesboro, TN). The resulting DNA samples were incubated overnight at 55°C in a lysis buffer containing proteinase K and used as a template DNA for PCR analysis after heat inactivation of proteinase K at 55°C for 15 min.

Genomic DNA was amplified and labeled with radioisotope by 35-40 cycles of PCR with a denaturation step at 94°C for 1 min, an annealing step of 55-62°C for 45-60 s, and an elongation step of 72°C for 1 min on a total of 5  $\mu$ l of reaction mixture [0.5  $\mu$ M of each primer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM each deoxynucleotide triphosphate, 0.5 U of *Taq* DNA polymerase, and 0.5  $\mu$ l of [ $\gamma$ -<sup>32</sup>P]dATP (10  $\mu$ Ci/ml)]. The PCR products were diluted with equal volumes of formamide-dye loading buffer, heated at 95°C for 10 min, and electrophoresed on an 8% polyacrylamide gel containing 7.5 M urea. The radioisotope-labeled microsatellite sequences were resolved by denaturing gel electrophoresis. Several exposures of each autoradiograph were performed to obtain an optimum range of allelic intensities for densitometric analyses. The PCR amplifications were repeated twice to ensure the reproducibility of results in cases where the bandshifts were not clearly informative in the first attempt.

**Microsatellite Markers and Analyses.** Allelic imbalances were measured by performing microsatellite analysis (MSI) on all matched normal and tumor

Table 2 Tumor DNA characteristics by stage

	Stages II & III		Stage II		Stage III		P
	n	%	n	%	n	%	
MSI	209		49		160		0.02
MSS/MSI-L	179	85.6	37	75.5	142	88.8	
MSI-H	30	14.4	12	24.5	18	11.3	
LOH (any)	209		49		160		0.10
Negative	102	48.8	29	59.2	73	45.6	
Positive	107	51.2	20	40.8	87	54.4	
LOH 17p	188		44		144		0.86
Negative	168	89.4	39	88.6	129	89.6	
Positive	20	10.6	5	11.4	15	10.4	
LOH 18q	208		48		160		0.27
Negative	129	62.0	33	68.8	96	60.0	
Positive	79	38.0	15	31.3	64	40.0	
LOH on 17p among all LOH tumors	96		17		79		0.34
Negative	76	79.2	12	70.6	64	81.0	
Positive	20	20.8	5	29.4	15	19.0	
LOH on 18q among all LOH tumors	107		20		87		0.81
Negative	29	27.5	5	25.0	24	28.0	
Positive	78	72.5	15	75.0	63	72.0	
TGFβRII mutations	200		47		153		0.01
Negative	183	91.5	39	83.0	144	94.1	
Positive	17	8.5	8	17.0	9	5.9	
TGFβRII mutations in MSI-H tumors	30		12		18		0.30
Negative	16	53.3	5	41.7	11	61.1	
Positive	14	46.7	7	58.3	7	38.9	

tissues by PCR amplification. A panel of 11 microsatellite markers, comprising 8 dinucleotide repeats (D2S123, D5S346, D17S250, D3S1029, D17S261, D18S64, D18S69, and D18S474), 2 mononucleotide repeats (BAT25 and BAT26), and 1 tetranucleotide repeat (MYCL1), were used to determine tumor MSI status. These markers included the recommended reference panel of five markers for the detection of MSI proposed at the National Cancer Institute collaborative meeting on MSI in colorectal cancer (28). Loci were scored according to the guidelines published.

Changes in the electrophoretic mobility of DNA amplified by PCR were used to assess the MSI. Tumors with a shift in at least two of the five recommended markers were classified as MSI-H, in accordance with the international criteria (28). MSI-L was defined as a shift in only one of the five markers. Tumors not showing allelic shifts were termed MSS and in this study were categorized along with MSI-L tumors for all statistical purposes. Scoring of the MSI was undertaken independently by two authors (A. G. and C. A.), who also arrived at a consensus in discrepant cases.

The presence of mutations of the poly(A)<sub>10</sub> tract of TGFβRII, poly(G)<sub>8</sub> tract of IGFIR, poly(G)<sub>8</sub> tract of BAX, poly(A)<sub>8</sub> tract of hMSH3, and poly(C)<sub>8</sub> tract of hMSH6 was investigated by PCR followed by PAGE as described above. The presence of bandshifts or an additional band was interpreted as a mutation (17).

**LOH Analysis.** Seven sets of polymorphic microsatellite sequences that are tightly linked to known tumor suppressor genes and DNA MMR genes were used to identify significant allelic losses in the carcinoma specimens. DNA was amplified by PCR using <sup>32</sup>P-end-labeled primers at microsatellite loci linked to the hMSH2 locus on 2p16 (D2S123), hMLH1 locus on 3p23-21.3 (D3S1029), APC locus on 5q21 (D5S346), p53 locus on 17p13 (D17S261), and DCC/SMAD2/SMAD4 region on 18q21.3 (D18S64, D18S69, and D18S474). Assessment of LOH was assigned when a tumor allele showed at least a 50% reduction in the relative intensity of one allele in neoplastic tissue compared with the matched normal DNA.

**Statistical Methods.** MSI and LOH at each locus were assessed for potential associations with a number of clinicopathological parameters, including tumor stage (stages 2 or 3), age at diagnosis of the disease (years), tumor location (proximal, including cecum, right colon, hepatic flexure, and transverse colon; distal, including splenic flexure, left colon, sigmoid colon, and rectosigmoid), differentiation (poor, moderate, or well), histology (adenocarcinoma, colloid, signet ring, or other), nodal status (0, ≥1 and <3, or ≥4), gender (male or female), and race (white or other). The amount of missing data varied. Univariate associations of baseline prognostic variables were assessed

using the  $\chi^2$  test or Fisher's exact test as appropriate. The Kaplan-Meier method was used to estimate disease-free and overall survival. Differences between groups were evaluated using the log-rank test. The simultaneous prognostic effect of various factors was determined by multivariate analysis using Cox's proportional-hazards models. All reported *P*s are two-sided, and *P* < 0.05 is considered significant.

## RESULTS

**Tumor Characteristics.** The study cohort contained 49 stage II and 160 stage III colorectal cancers. There was a significant difference in the proportions of well, moderate, and poorly differentiated tumors by stage (*P* = 0.03). Overall, 19.3% of the tumors studied were poorly differentiated, with a higher proportion of poorly differentiated tumors in the stage III group (12.8% versus 21.3%; Table 1). The tumors were evenly distributed among proximal (47%) and distal (53%) sites, with no difference in tumor location between the stage II and stage III groups (*P* = 0.40). The majority of patients with stage III tumors had limited nodal involvement, with tumor found in three or fewer nodes in 70% of these cases.

**Microsatellite Analysis of Tumors.** MSI analysis was performed using paraffin-embedded materials from 209 tumors and either normal colonic mucosa or uninvolved lymph nodes from the same patient. Informative results were obtained for all of the tumors (Table 2). Of the 209 cases, 136 (65%) were MSS, 43 (21%) were MSI-L, and 30 (14%) were MSI-H. By stage, 28 (57%) of the stage II tumors were MSS, whereas 9 (18%) were MSI-L and 12 (25%) were MSI-H. Of the stage III cases, 108 (68%) were MSS, 34 (21%) were MSI-L, and 18 (11%) showed MSI-H. The proportion of MSI-H tumors was higher in stage II than in stage III patients (*P* = 0.02; Table 2). MSI-H tumors were more likely to be poorly differentiated (*P* = 0.02) and were also more commonly found in cases without nodal tumor involvement (*P* = 0.03; Table 3).

**Assessment of LOH.** We identified 107 tumors (51%) with LOH at one or more of the eight loci studied (Table 2). LOH events were present in 41% of the stage II and 55% of the stage III patients. Overall, the frequency of LOH in tumors with any LOH event was most common on 18q (72.5% of 107 for D18S64, D18S69, and D18S474), followed by 5q (22% of 103 for D5S346), 17p (21% of 96

Table 3 MSI status: correlates with disease characteristics

	Cohort		MSS/MSI-L		MSI-H		P
	n	%	n	%	n	%	
Age (yrs)	209		179		30		0.68
<50	33	15.8	27	15.1	6	20.0	
50-65	86	41.1	73	40.8	13	43.3	
>65	90	43.1	79	44.1	11	36.7	
Gender	209		179		30		0.63
Male	117	56.0	99	55.3	18	60.0	
Female	92	44.0	80	44.7	12	40.0	
Race	208		178		30		0.59
White	174	83.7	150	84.3	24	80.0	
Other	34	16.3	28	15.7	6	20.0	
Differentiation	202		173		29		0.02
Well	26	12.9	23	13.3	3	10.3	
Moderate	137	67.8	122	70.5	15	51.7	
Poor	39	19.3	28	16.2	11	37.9	
Lymph nodes	205		175		30		0.03
0	49	23.9	36	20.6	13	43.3	
1-3	109	53.2	97	55.4	12	40.0	
>4	47	22.9	42	24.0	5	16.7	
Tumor site <sup>a</sup>	205		175		30		0.71
Proximal	96	46.8	81	46.3	15	50.0	
Distal	109	53.2	94	53.7	15	50.0	
TGFβRII mutations	200		170		30		<0.0001
Negative	183	91.5	167	98.2	16	53.3	
Positive	17	8.5	3	1.8	14	46.7	

<sup>a</sup> Four patients had multiple tumor sites and were excluded from the analysis.

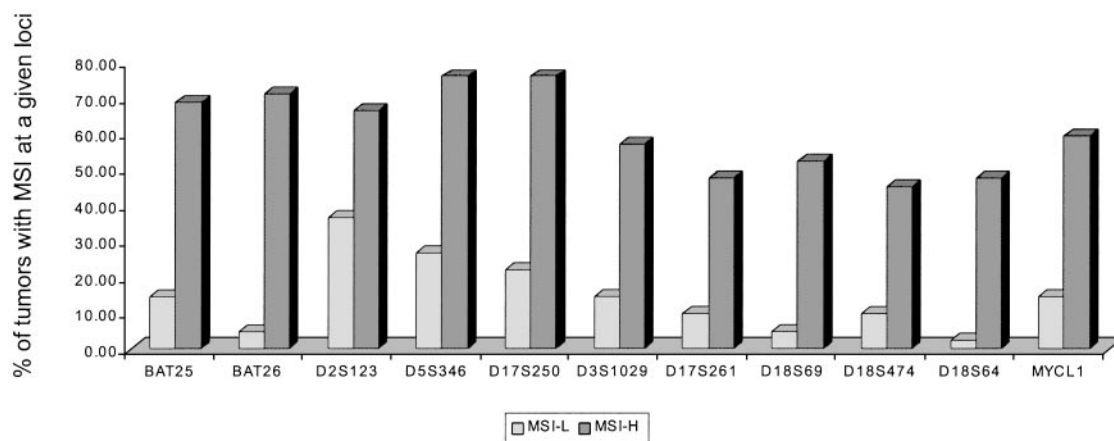


Fig. 1. MSI determination for 209 sporadic colon cancers. Tumor MSI status was assessed by examining 12 microsatellite markers, including 8 dinucleotide repeats (*D2S123*, *D5S346*, *D17S250*, *D3S1029*, *D17S261*, *D18S64*, *D18S69*, and *D18S474*), 2 mononucleotide repeats (*BAT25* and *BAT26*), and 1 tetranucleotide repeat (*MYCL1*).

for *D17S261*), 3p (14% of 98 for *D3S1029*), 2p (10% of 101 for *D2S123*), and 1p (6% of 107 for *MYCL1*), respectively (Fig. 1). The overall frequency of LOH at 17p was 11% for all tumors and was equally distributed among stage II and stage III cancers (11 and 10%, respectively;  $P = 0.86$ ). The 18q locus showed allelic losses in 38% of cases, with 31% of the stage II tumors and 40% of the stage III tumors positive for LOH on 18q ( $P = 0.27$ ; Table 2).

**Target Gene Mutations.** Tumors were evaluated for mutations in the mononucleotide repeat regions of *TGFβRII*, *IGFIIR*, *BAX*, *hMSH3*, and *hMSH6*. These genes are commonly mutated in sporadic MSI-H colorectal cancers as well as in HNPCC-associated tumors because of the loss of a functional MMR system. Target gene mutations occurred almost exclusively in MSI-H tumors. Overall, *TGFβRII* mutations were seen in 8.5% of all tumors but in 47% of MSI-H cancers (Table 4). Similarly, mutation frequencies of 6.7% in *IGFIIR*, 28% in *BAX*, 17% in *hMSH3*, and 10% in *hMSH6* were observed in MSI-H tumors (Table 4). Although few in number in patients with MSI-H tumors, mutations in *TGFβRII* were seen more frequently in stage II cancers ( $P = 0.01$ ; Table 2). We were not able to establish this difference in MSI-H tumors because of the small number of tumors that were MSI-H ( $P = 0.30$ ; Table 2).

The great majority of the target gene mutations identified were in MSI-H tumors, with the exceptions that 1 of 175 MSS/MSI-L tumors contained *IGFIIR* bandshifts and 3 of 170 MSS/MSI-L cancers contained *TGFβRII* mutations. Interestingly, only one bandshift in the target genes examined was found in the 100 cancers that were MSS/MSI-L and also had one or more LOH events.

Table 4 Target gene mutations

	MSS		MSI-L		MSI-H	
	n	%	n	%	n	%
<i>TGFβRII</i>	129		41		30	
Mutation present	2	1.6	1	2.4	14	46.7
Mutation absent	127	98.4	40	97.6	16	53.33
<i>IGFIIR</i>	133		42		30	
Mutation present	0	0.0	1	2.3	2	6.7
Mutation absent	133	100.0	41	97.7	28	93.3
<i>BAX</i>	132		41		29	
Mutation present	0	0.0	0	0.0	8	27.6
Mutation absent	132	100.0	41	100.0	21	72.4
<i>MSH3</i>	126		40		29	
Mutation present	0	0.0	0	0.0	5	17.2
Mutation absent	126	100.0	40	100.0	24	82.8
<i>MSH6</i>	136		43		30	
Mutation present	0	0.0	0	0.0	3	10.0
Mutation absent	136	100.0	43	100.0	27	90.0

Table 5 Overlap between LOH and MSI events in colorectal cancers

A. LOH by MSI status							
	LOH present		LOH absent				
	n	%	n	%			
MSS	70	65.4	66	64.7			
MSI-L	30	28.0	13	12.7			
MSI-H	7	6.6	23	22.5			
Total	107		102				
	LOH	1p	2q	3p	5q	17p	18q
LOH (n = 107)							
n	7	10	14	23	20	78	
%	6%	10%	14%	22%	21%	72%	
MSS (n = 136)							
n	70	7	4	8	19	14	50
%	51.5%	5%	3%	6%	14%	10%	37%
MSI-L (n = 43)							
n	30	0	6	5	4	4	23
%	69.8%	0%	14%	11%	9%	9%	53%
MSI-H (n = 30)							
n	7	0	0	1	0	2	5
%	23.3%	0%	0%	3%	0%	7%	16%
B. MSI (nucleotide classification) by LOH status							
	Cohort		LOH present		LOH absent		P
	n	%	n	%	n	%	
MSI	73		37		36		0.0004
Mono	11	15.1	7	18.9	4	11.1	
Di	41	56.2	27	73.0	14	38.9	
Both	21	28.8	3	8.1	18	50.0	

**Overlap of Different Pathways of Genomic Instability.** We next investigated the degree of overlap between tumors with LOH and those with MSI. Of the 107 tumors with LOH, 65.4% were MSS, 28.0% were MSI-L, and 6.6% were MSI-H (Table 5). Of 136 MSS tumors, 51.5% also contained an LOH event at one or more of the loci tested, as did 69.8% of the MSI-L tumors and 23.3% of the MSI-H tumors. The proportions of LOH-positive tumors differed significantly by MSI status, with a higher observed frequency of LOH among MSI-L tumors ( $P = 0.0005$ ).

Studies evaluating the clinical behavior of MSS versus MSI-H colon cancers suggest that MSI-L tumors are more similar to MSS cancers than they are to tumors with high levels of MSI (29). The genomic consequences of MSI-L status are unknown. We examined the distribution of cancers that were MSS or MSI-L tumors without LOH, MSS or MSI-L tumors with LOH, MSI-H tumors without LOH, and MSI-H tumors with LOH (Fig. 2). We found that 3.4% of all

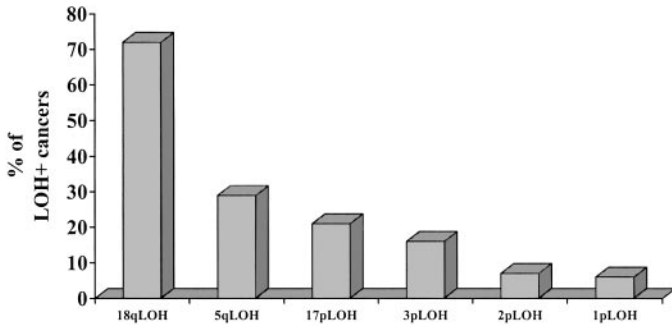


Fig. 2. LOH at specific loci among tumors with any LOH event. LOH events identified in 107 of 209 sporadic colon cancers. Markers used were D18S64, D18S69, and D18S474 (18q); D5S346 (5q); D17S261 (17p); D3S1029 (3p); D2S123 (2p); and MYCL1 (1p).

tumors showed MSI-H and had LOH, 11% were MSI-H and negative for LOH, whereas 47.8% of the tumors were MSS or MSI-L and showed LOH on any of the loci tested. Interestingly, we identified a group of 37.8% of all cancers examined that were either MSS or MSI-L and had no LOH events and therefore did not demonstrate signs for either of the two pathways of genomic instability (Fig. 3). Comparisons of the clinical and pathological variables of these four tumor categories are provided in Table 6.

## DISCUSSION

During colorectal carcinogenesis, epithelial cells in the large intestine acquire the characteristics of malignant behavior, which are defined, at a minimum, by the ability to invade the underlying basement membrane. This multifactorial process involves the accumulation of alterations in DNA structure that contribute to the loss of genes controlling cell proliferation and migratory capacity (1). A wealth of data supports the conclusion that multiple genetic events are required for the development of invasive carcinoma (30–32). Our current understanding of intestinal carcinogenesis suggests that at least two mechanisms are capable of producing the mutations required for a cell to demonstrate a malignant phenotype. These mechanisms include CIN, characterized in tumor DNA by the presence of multiple LOH events, and loss of MMR function, which is defined by high levels of MSI. In this study, we examined a large number of sporadic colon cancers to determine whether these two mechanisms of tumorigenesis are independent or whether there exists a significant degree of overlap.

MSI in this study was defined by the commonly applied internationally accepted criteria for identifying MMR defects (33). To be scored as MSI-H, bandshifts indicating alterations in tumor DNA microsatellite size must have been detected at two or more of the five standard markers. Data from multiple sources support the use of these five loci as a working definition of MSI-H (28). We also examined six additional microsatellites, including MYCL1, D3S1029, D17S261, D18S69, D18S474, and D18S64. When we considered all 11 microsatellite sites and defined tumors as MSI-H when 20% of the sites showed MSI, we confirmed that the standard five markers alone detected each and every MSI-H cancer.<sup>7</sup> To investigate allelic losses by LOH, we used eight polymorphic markers mapped closely to key tumor suppressor genes that are believed to be lost during colon carcinogenesis (34). Detection of LOH at one or more of these sites was taken as evidence of loss of tumor suppressor activity by CIN. It is possible and even likely that additional tumors would be reclassified by the addition of markers outside of the usual deletion sites. The point to be made, however, is that without a single LOH event at the eight sites examined, it is highly unlikely that a tumor exhibits the

widespread LOH that is characteristic of tumors arising in the setting of CIN. With these data, we classified the cancers as belonging to one of four genotypes: MSI-H without LOH; MSI-H with LOH; MSS/MSI-L with LOH; or MSS/MSI-L without LOH.

MSI-H without evidence for CIN was found in 11% of the cancers analyzed. MSI-H is caused by a defect in DNA MMR capability, which most commonly is achieved by hypermethylation of the hMLH1 promoter (35). Because our study population excluded patients with HNPCC, it is unlikely that a significant proportion of the MSI-H cancers were caused by germ-line mutation of MMR genes. Cancers associated with MMR defects are typically diploid, although comparative genomic hybridization shows that these tumors may also demonstrate amplifications and deletions of single alleles or chromosomes (36). In agreement with this, 3.4% of the cancers in our study showed the coincidence of MSI-H and LOH events. It is possible that loss of MMR function in this subset was caused by allelic loss of one of the major MMR genes, such as hMLH1 or hMSH2. We found no instances of LOH at 2p in MSI-H cancers, and only one of the MSI-H tumors showed LOH at 3p in a region suggesting hMLH1 loss (Table 5). Interestingly, we found that tumors with mononucleotide repair deficiency only were almost equally likely to be LOH positive or negative, whereas tumors with dinucleotide instability were more likely to exhibit LOH and tumors with both were less likely to exhibit LOH ( $P = 0.0004$ ).

We therefore hypothesize that, for MSI-H tumors, the main force driving mutation acquisition is not associated with allelic losses at the MMR loci and that the rare associated LOH events are caused by a general genomic instability that is typical for these neoplasms (37). Interestingly, we observed that the frequency of LOH events was significantly greater in tumors with MSI-L compared with MSI-H cancers. These data support recent studies suggesting that the molecular profiles of MSS and MSI-L tumors are indistinguishable (38, 39). Our data also support earlier work showing that MSI-H tumors differ from MSS/MSI-L cancers in histology and, possibly, in clinical behavior. MSI-H tumors are generally more likely to be poorly differentiated (40) but may also exhibit a less aggressive clinical behavior (28). Examination of treatment outcomes for this relatively small and nonuniform cohort did not show a survival advantage for MSI-H cancers, although we did find significantly increased disease-free

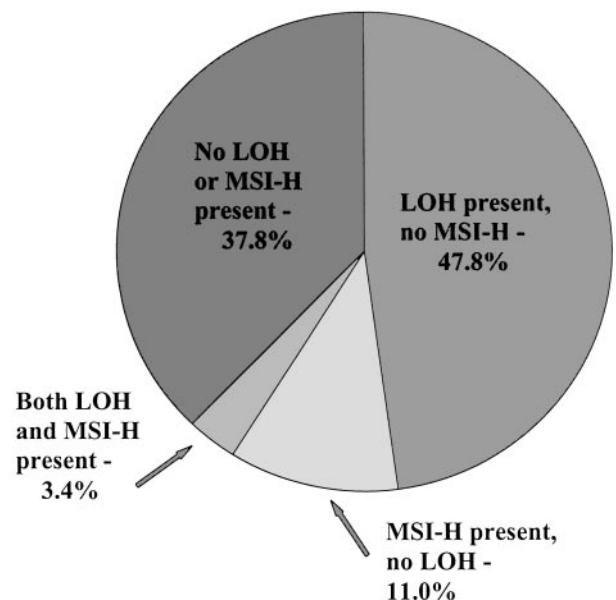


Fig. 3. Exclusiveness and overlap among subsets of genomic instability. Summary of genomic instability patterns for 209 sporadic colon cancers.

<sup>7</sup> C. N. Arnold, A. Goel, J. M. Carethers, L. Wasserman, C. Compton, D. Niedzwiecki, R. J. Mayer, M. M. Bertagnolli, and C. R. Boland, unpublished data.

Table 6 Outcome data: cohort tumor characteristics

	Cohort		MSI-H, LOH present		MSI-H, no LOH		MSS/MSI-L, LOH present		MSS/MSI-L, no LOH		P
	n	%	n	%	n	%	n	%	n	%	
Age (yrs)	209		7		23		101		78		0.55
<50	33	15.8	0	0.0	6	26.1	17	16.8	10	12.8	
50-65	86	41.1	4	57.1	9	39.1	37	36.6	36	46.2	
>65	90	43.1	3	42.9	8	34.8	47	46.5	32	41.0	
Gender	209		7		23		101		78		0.47
Male	117	56.0	6	85.7	12	52.2	55	54.5	44	56.4	
Female	92	44.0	1	14.3	11	47.8	46	45.5	34	43.6	
Race	208		7		23		101		77		0.72
White	174	83.7	6	85.7	18	78.3	87	86.1	63	81.8	
Other	34	16.3	1	14.3	5	21.7	14	13.9	14	18.2	
Differentiation	202		7		22		96		77		0.03
Well	26	12.9	0	0.0	3	13.6	10	10.4	13	16.9	
Moderate	137	67.8	6	85.7	9	40.9	72	75.0	50	64.9	
Poor	39	19.3	1	4.4	10	45.5	14	14.6	14	18.2	
Stage	209		7		23		101		78		0.07
Stage II	49	23.4	2	28.6	10	43.5	18	17.8	19	24.4	
Stage III	160	76.6	5	71.4	13	56.5	83	82.2	59	75.6	
Lymph nodes	205		7		23		99		76		0.16
0	49	23.9	3	42.9	10	43.5	17	17.2	19	25.0	
1-3	109	53.2	3	42.9	9	39.1	59	59.6	38	50.0	
>4	47	22.9	1	14.3	4	17.4	23	23.2	19	25.0	
Tumor site	205		7		23		98		77		0.07
Proximal	96	46.8	3	42.9	12	52.2	37	37.8	44	57.1	
Distal	109	53.2	4	57.1	11	47.8	61	62.2	33	42.9	

survival and overall survival for the subset of patients with stage II disease and MSI-H cancers (data not shown).

CIN, as evidenced by allelic loss at one or more of the eight markers tested, was observed in 51.2% of the cancers examined. Tumors that exhibited LOH without high-level MSI (no MSI-H) comprised 47.8% of the cancers. The presumed course of tumor progression in this subset involves accumulated allelic losses at tumor suppressor loci (1). The processes responsible for CIN are unknown. Many mechanistic explanations have been offered for CIN, but none has been shown to be sufficient to account for this process in colorectal cancer. One explanation involves *hBUB1*, a protein that regulates the G<sub>2</sub>-M cell cycle checkpoint. Mutation of this gene may disturb the spindle checkpoint in a dominant-negative manner and therefore provide one of the molecular events leading to CIN. Such mutations, however, are rarely found in colorectal cancers (4). Others have recently suggested that the *hSecurin* gene is required for chromosomal instability because it is involved in chromosomal segregation during anaphase and is observed to be modified in neoplasms with CIN (41). Another possibility relates to APC loss, an event that generally

occurs early in tumorigenesis. In addition to regulating Wnt pathway signaling, APC modulates cytoskeletal activity and may directly influence kinetochore function (42). It is likely that multiple causes of CIN will be discovered through the study of these and other events in early carcinogenesis.

The most intriguing subgroup identified in this study was the nearly 38% of colon cancers that lacked any evidence of either CIN or MSI. The likelihood of misclassification of this group is small because a many markers were used to identify LOH and MSI events. The molecular events leading to the development of cancers in this subgroup with no signs of genomic instability are unknown. One reasonable possibility is the transcriptional silencing of growth and differentiation genes by epigenetic modification. Aberrant promoter methylation, leading to loss of tumor suppressor function, has been observed in a variety of cancers (43). Epigenetic modification of tumor suppressor genes is characteristically age dependent (44), and there are several reports in the literature on tumor-specific methylation patterns (45). One example already mentioned is the age-associated loss of *hMLH1* by promoter hypermethylation

Table 7 Participating Institutions

Institution name	Location	Principal Investigator	Supported by Grant
CALGB Statistical Office	Durham, NC	Stephen George, Ph.D	CA33601
Dana Farber Cancer Institute	Boston, MA	George P. Canellos, M.D.	CA32291
Dartmouth Medical School-Norris Cotton Cnrc Ctr	Lebanon, NH	Marc Ernstoff, M.D.	CA04326
Massachusetts General Hospital	Boston, MA	Michael L. Grossbard, M.D.	CA12449
Mount Sinai School of Medicine	New York, NY	Lewis Silverman, M.D.	CA04457
Rhode Island Hospital	Providence, RI	William Sikov, M.D.	CA08025
Roswell Park Cancer Institute	Buffalo, NY	Ellis Levine, M.D.	CA02599
Southeast Cancer Control Consortium Inc. CCOP	Goldsboro, NC	James N. Atkins, M.D.	CA45808
SUNY Upstate Medical University	Syracuse, NY	Stephen L. Graziano, M.D.	CA21060
The Ohio State University	Columbus, OH	Clara D. Bloomfield, M.D.	CA77658
University of California at San Diego	San Diego, CA	Stephen Seagren, M.D.	CA11789
University of California at San Francisco	San Francisco, CA	Alan Venook, M.D.	CA60138
University of Chicago Medical Center	Chicago, IL	Gini Fleming, M.D.	CA41287
University of Illinois at Chicago	Chicago, IL	David Gustin, M.D.	CA74811
University of Iowa	Iowa City, IA	Gerald Clamon, M.D.	CA47642
University of Maryland Cancer Center	Baltimore, MD	David Van Echo, M.D.	CA31983
University of Massachusetts Medical Center	Worcester, MA	Mary Ellen Taplin, M.D.	CA37135
University of Minnesota	Minneapolis, MN	Bruce A. Peterson, M.D.	CA16450
University of Missouri/Ellis Fischel Cancer Center	Columbia, MO	Michael C. Perry, M.D.	CA12046
University of North Carolina at Chapel Hill	Chapel Hill, NC	Thomas C. Shea, M.D.	CA47559
University of Tennessee Memphis	Memphis, TN	Harvey B. Niell, M.D.	CA47555
Wake Forest University School of Medicine	Winston-Salem, NC	David D. Hurd, M.D.	CA03927
Walter Reed Army Medical Center	Washington, DC	John C. Byrd, M.D.	CA26806

tion that is found in some colorectal cancers (44). Additional genes relevant to colorectal carcinogenesis that are sensitive to methylation-related silencing include *APC*, *p16*, *IGFII*, *MyoD*, and the gene encoding the estrogen receptor (32, 45). We are in the process of further characterizing the MSS/MSI-L without LOH subset, and in support of this hypothesis, we have observed promoter methylation of multiple tumor suppressor genes.<sup>8</sup>

The primary goal of our study was to identify which proportion of colon cancers exhibited the standard characteristics of either MSI or CIN. Long-term clinical outcome data were available for these patients; the cohorts were too small and heterogeneous, however, to draw definite conclusions as to whether the presence or absence of MSI or CIN separated patients into distinct prognostic categories. This issue will require future study in better-defined patient populations.

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