Published in final edited form as:
Dis Colon Rectum. 2009 July ; 52(7): 1304–1311. doi:10.1007/DCR.0b013e3181a0e5df.

A comparison of colon and rectal somatic DNA alterations

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Abstract

Background and Aims—Differences in acquired mutations in colon and rectal tumors may account for differences in risk factors. In this study, we examined similarities and differences in somatic alterations in colon and rectal tumors.

Methods—Cases were identified from two large population-based case-control studies of colon cancer and rectal cancer. We sequenced exons 5 to 8 of the *p53* gene and codons 12 and 13 of the Ki-*ras* gene to identify tumor mutations. Micro-satellite instability was determined based on *BAT26* and *TGFβRII* analysis; CpG Island Methylator Phenotype was determined based on having two or more of the following markers methylated *p16*, *MLH1*, MINT1, MINT2, and MINT31.

Results—*p53* mutations were observed in 39.7 percent of proximal, 51.0 percent of distal, and 46.6 percent of rectal tumors; Ki-*ras* mutations were observed in 36.0 percent of proximal, 26.9 percent of distal and 30.5 percent of rectal tumors. While 40.9 percent of proximal tumors were considered CpG Island Methylator Phenotype positive (having 2 or more of 5 markers methylated), only 12.9 percent of distal and 11.9 percent of rectal tumors were CpG Island Methylator Phenotype positive. Likewise, microsatellite instability was observed in 23.7 percent of proximal and only 3.8 percent of distal and 2.0 percent of rectal tumors. Over 50 percent of distal colon or rectal tumors had only one acquired mutation, while only 35.1 percent of proximal tumors had one mutation. The most common single mutation for colon and rectal tumors was *p53* followed by Ki-*ras* mutations.

Conclusions—Our findings suggest unique mutational pathways are involved in the development of most colorectal tumors. Proximal colon cancers are more likely to have microsatellite instability, CpG Island Methylator Phenotype, and Ki-*ras* mutations, while rectal and distal colon tumors are more likely to have a *p53* mutation than proximal colon tumors. Overall, rectal and distal colon tumors share similar mutational frequencies than do proximal colon tumors.

Keywords

Colon cancer; Rectal cancer; *p53*; Ki-*ras*; CpG Island Methylator Phenotype; microsatellite instability; Distal; Proximal

No Conflict of Interest exists for any of the authors

No reprints are available

Introduction

The belief that colorectal cancer is a heterogeneous disease stems from several avenues of observation. Numerous studies have shown that risk factors associated with colon and rectal cancers differ by tumor site [1-6]. For instance, although physical activity has been consistently associated with reduced risk of colon cancer, associations are not consistent for rectal cancer [4, 7, 8]; obesity appears to influence risk of colon but not rectal cancer [5, 9]. Dietary associations influence risk differently based on tumor site [10, 11]. Furthermore, men are more likely to be diagnosed with rectal tumors than women. People diagnosed with rectal cancer are slightly younger than those diagnosed with colon cancer despite the fact that reported family history of colorectal cancer is less common among people diagnosed with rectal cancer than with colon cancer [1]. Familial syndromes such as HNPCC, *APC*, and attenuated *APC* cluster in the proximal area of the colon rather than the distal colon [12-14]. These syndromes are rare and combined estimates of their occurrence range from one to five percent of cases [15]. Evaluation of colon tumor biomarkers shows that microsatellite instability and CpG island methylator phenotype (CIMP) are predominately characteristics of tumors that are more proximally located [16, 17]. Additionally, it has been suggested that distal and rectal tumors are similar in occurrence of *p53* mutations [18].

Colon tumor mutations also have been associated with specific diet and lifestyle factors, studies of rectal cancer specifically are generally lacking. Studies have documented unique associations between smoking cigarettes and MSI and CIMP in colon tumors [19, 20], dietary intake has been associated with specific Ki-ras mutations and p53 mutations [21-23], and alcohol use has been associated with MSI tumors [24]. Given the associations between diet and lifestyle factors and colon tumors, it is possible that differences in prevalence of tumor mutations observed for colon and rectal cancers contribute to differences in observed risk factors for these two disease sites.

The purpose of this report is to describe the tumor characteristics from two population-based collections of colon and rectal tumors that include information on *p53*, Ki-*ras*, MSI, CIMP, and *BRAF* mutations. We provide details of specific tumor marker distribution based on tumor site and gender. While previous studies have shown differences in some of these mutations by site, this large population-based study adds to and confirms other reports.

Methods

Comparison data come from participants identified for a case-control study of colon cancer or from a case-control study of rectal cancer conducted in the Kaiser Permanente Medical Care Program of Northern California (KPMCP) and the state of Utah. All eligible cases within these defined areas were identified and recruited for the study which involved a detailed in-person interview and a blood draw. Case eligibility was determined by the Surveillance Epidemiology and End Results (SEER) Cancer Registries in Northern California and in Utah. To be eligible for these studies, participants had to be between 30 and 79 years of age at time of diagnosis, English speaking, mentally competent to complete the interview, could not have had previous colorectal cancer [25], and could not have known (as indicated on the pathology report) familial adenomatous polyposis, ulcerative colitis, or Crohn's disease.

First primary colon cancer (ICD-O 2nd edition codes 18.0, 18.2 to 18.9) diagnosed between October 1, 1991 and September 30, 1994 in Northern California and Utah were eligible for the study; tumor blocks were obtained and genetic analyses completed between 1995 and 1999. Of 1917 colon cancer cases eligible for the study from Northern California and Utah, we extracted DNA on 1637 or 85 percent [26]. Cases with a first primary tumor in the recto-sigmoid junction or rectum were identified between May 1997 and May 2001; tumor block

ascertainment and genetic analyses were completed in 2007. Of the 1265 cases who consented to having their tissue released, we obtained DNA from 1022 cases (81 percent of cases). Of the 234 cases that we did not make DNA, 27 did not have a block available; we did not receive the block for 132, and we were unable to make DNA from the block received for 75.

Block retrieval involved obtaining biopsy prior to treatment as well as paraffin embedded tissue from the resection. In some instances, because of radiation prior to resection for rectal cases, tissue was limited from the resection and therefore, biopsy specimens were used for making tumor DNA. In Utah, blocks were requested for all cases except those who refused release of blocks. For those who were not interviewed and had not signed a medical record release, the Utah Cancer Registry retrieved the blocks and released them to the study without key identifiers of name, address, and complete date of birth (year and month of birth were released). At the KPMCP, samples were retrieved from persons who signed a consent form or who had died. Detailed methods for collection of tissue have been described [26].

Genetic Analysis

Genetic analyses were conducted on colon blocks between 1995 and 1999; similar analyses were performed on the rectal blocks analyzed between 2001 and 2007. Tumor DNA was obtained from paraffin-embedded tissue as described previously. Tumors were characterized by their genetic profile that include sequence data for exons 5 through 8, or the hotspots of the *p53* gene; sequence data for Ki-*ras* codons 12 and 13; five CpG Island markers MINT1, MINT2, MINT31, *p16*, and *MLH1*; the V600E *BRAF* mutation, and microsatellite instability (MSI) as determined by *BAT26* and *TGF*β*RII* 17, [21, 27, 28]. Although this MSI assessment for this study preceded the Bethesda consensus panel, *BAT26*, a mononucleotide repeat is by itself a very good measure of generalized instability. *BAT26* and *TGF*β*RII* show a high correlation with the Bethesda consensus panel [29]. Using a hierarchical approach, we have shown that 95 percent of tumors can be classified as either stable or unstable using *BAT26* alone. At this time there is no “consensus” as to the appropriate CpG island panel or method of detection to determine CIMP. However, we have used our panel to demonstrate significant relationships between CIMP and numerous clinicopathologic variables, including cigarette smoking and the *BRAF* V600E mutation, which were independent of microsatellite instability [17, 20] This work has helped to support the legitimacy of the CIMP concept [30]. Germline DNA obtained from normal tissue in the paraffin blocks or from blood samples provided by study participants was used to verify that mutations were acquired rather than inherited.

Statistical analysis

The distribution of genetic alterations in *p53*, Ki-*ras*, *BRAF* V600E mutations; CIMP and MSI status were compared between colon and rectal and by proximal and distal colon site using a Mantel-Haenszel chi-squared test statistic. Proximal tumors were defined as tumors in the cecum through the transverse colon; distal tumors were those from the splenic flexure through the sigmoid colon; rectal cancer was defined as tumors located in the rectosigmoid junction or rectum. In addition to *p53* or Ki-*ras* mutation status, tumors were also assessed as to specific types and locations of *p53* or Ki-*ras* mutations. Data were analyzed by age (30 to 64 years and 65 to 79 years), and gender. Because associations did not differ by either age or gender, data are presented for the entire study population.

To help visualize the carcinogenic pathways for proximal, distal and rectal tumors, we used un-timed oncogenetic trees as described by Desper *et al.* in 1999 [31]. An oncogenetic tree is a rooted directed tree with edges pointing away from the root [31, 32]. The root presents the state of tissue with none of the measured somatic alterations. Each of the other nodes is associated with a particular somatic alteration. According to the oncogenetic tree model, for an alteration to occur in a particular tumor, all of the alterations corresponding to nodes that lie on the

directed path from the root to the corresponding node must also occur in the tumor. As a consequence, each tumor corresponds to a "subtree" of the oncogenetic tree containing all the alterations in the tumor. Each edge is labeled by the "transition probability" - the conditional probability that a tumor will acquire the alteration corresponding to the next node away from the root, given that the alteration corresponding to the node proximal to the root is already present. When two edges emanate from the same node, neither of the alterations on each edge is required before the other can occur. The method used to fit the oncogenetic tree is described in greater detail elsewhere [33, 34].

The oncogenetic trees, together with edge labels, were constructed using a simple algorithm. The parent of each node was found by maximizing the weight function function $w\left(v_i, v_j\right) = \log \frac{p_{ij}}{p_i\left(p_i + p_j\right)}$. If the true oncogenetic tree is not skewed, a concept first described in Desper *et al.* 31, the algorithm is guaranteed to reconstruct the correct tree. A nonparametric bootstrap re-sampling method was used to estimate the amount of sampling variability in the fitted tree. The bootstrap method samples from the rows of the data with replacement N times, where N is the size of the original sample [35]. An oncogenetic tree is fit to the resampled data. The procedure is repeated M times. We repeated the procedure M = 1000 times.

Results

The distribution of tumor mutations for proximal colon, distal colon, and rectal tumors is shown in Table 1. Significant differences between sub-sites of proximal colon, distal colon, and rectal cancer were identified. CIMP+, MSI+, and *BRAF* mutated tumors occurred with a much greater frequency in proximal colon tumors than in either distal colon or rectal tumors. There were more similarities for prevalence of tumor mutations or epigenetic changes between tumors in distal colon and rectum than between tumors of the proximal and distal colon. Similar associations were observed when the data were examined by age at diagnosis and gender of the study participants (data not shown in table).

The specific types of *p53* (Table 2) and Ki-*ras* (Table 3) mutations observed were similar for all three sites when compared among those with a *p53* or Ki-*ras* mutation. Only when examining the *p53* hot-spots did we observe a significant difference, where missense mutations in codon 175 resulting in an amino acid change from arginine (R) occurred more frequently in rectal tumors than in colon tumors. With respect to *p53* hot-spots, proximal and distal colon tumors were more similar to each other than distal colon and rectal tumors (Table 2). Examination of Ki-*ras* mutations (Table 3) showed that rectal tumors were more likely to have mutations in codons 12 and 13 than either proximal or distal colon tumors.

Examination of the prevalence of CIMP, MSI, and *BRAF* alterations showed differences for CIMP overall as well as for specific CIMP markers, the number of CIMP markers methylated, and CIMP+ tumors with and without MSI and *BRAF* mutations (Table 4). Distal and rectal tumors were similar in all categories. CIMP+ proximal tumors contained a much higher proportion of MSI+ tumors (43.2 percent) than CIMP+ distal colon or rectal tumors (9.5 percent and 3.8 percent respectively).

The oncogenetic trees describe the probability of developing various mutations given the underlying system or root and any previously acquired mutations. Trees for proximal, distal, and rectal tumors were created separately. As shown in Figure 1, the estimated proportion of tumors that will develop Ki-*ras* mutations increases as one goes from proximal colon to rectal tumors. The estimated proportion of tumors that will develop a *p53* mutation is the same for rectal and distal colon tumors and almost twice that of proximal tumors. On the other hand, proximal tumors were more likely to have one or more MINT1, MINT2, or MINT31 (labeled

any MINT) marker methylated with subsequent paths that go the MSI and *MLH1* route and another that is more likely to move towards *p16* and BRAF mutations. Rectal tumors were least likely to have a MSI positive pathway if any MINT markers were methylated; methylated MINT markers were more likely to subsequently acquire a *p16*, BRAF and *MLH1* methylated marker. Bootstrap resampling of the oncogenetic trees consistently showed the same three major pathways for all trees generated. However, for proximal colon tumor, there was variation in the location of *BRAF*, where in some trees it followed the *p16* methylation and in others it followed the MSI and *MLH1* methylation path. Because of the rarity of BRAF mutations and MSI for both distal colon and rectal tumor sites, that arm of the tree was less stable following the ANY MINT pathway, where MSI and the methylation markers fell in different orders generating many unique trees.

Discussion

Our study corroborates previous reports of differences in tumor mutations observed for proximal and distal colon and rectal cancers [18, 36, 37]. Of note in our large population-based study, is the similarity in tumor marker distribution observed for rectal and distal colon tumors. While proximal tumors were more likely to have MSI or CIMP+, distal colon and rectal tumors were more likely to *p53* and Ki-*ras* mutations.

Defining disease pathways that lead to colorectal cancer is an ongoing concern. While early work by Vogelstein [38] suggested a sequence of mutational events that lead to colon cancer, evidence is accumulating that several mutational pathways are involved in the carcinogenic process [39]. Data presented here further support previous observations that multiple disease pathways of acquired DNA alterations are involved in developing colon and rectal tumors. Over 50 percent of distal and rectal tumors had a single somatic mutation and 34.6 percent of proximal tumors had a single mutation. Of those with only one mutation, the majority were *p53* mutations followed by Ki-*ras* mutations. While common multiple mutations for proximal tumors included MSI and CIMP or *BRAF*, a subset of CIMP+ tumors with *p53* and Ki-*ras* were observed, suggesting unique disease pathways. Studies have also shown inverse relationships between MSI and *p53* and Ki-*ras* mutations [29] Previous studies have reported that some risk factors may be associated with specific mutations. Of interest are the observations that cigarette smoking and alcohol intake are associated more strongly with MSI tumors [19, 24] than either Ki-*ras* or *p53*- mutated colon tumors. Examination of specific exposures and rectal tumor mutations will further our understanding of risk factors as well as the carcinogenic process.

Oncogenetic tree analysis allowed us to visually display probabilities of unique mutational pathways. These trees, although statistically generated, are extremely close to the actual proportion of tumors with specific mutations. Resampling and bootstrap methods were used to validate the tree structure and the three main branches were reproduced in all trees generated, suggesting stability to the three main colorectal mutation pathways. The shapes of the trees; i.e., the relationships among the alterations, were similar for all three sites; in all three trees, Ki-*ras* and *p53* mutations were not dependent on any other alteration, whereas the DNA methylation, MSI, and *BRAF* mutation consistently fell in the same pathway. It was the probabilities of the pathways that differed among the three site-specific trees. These oncogenetic trees reinforce the unique mutational pathways to colon and rectal cancer that exist and show the higher degree of similarity to distal and rectal tumors than to proximal and distal tumors.

Our findings have implications for future studies. Some epidemiologic and molecular studies examine associations with colon cancer which include both proximal and distal colon tumors and sometimes rectal tumors. Our data suggest that these tumor sites have different mutational characteristics. This is illustrated most vividly when looking at CIMP data, where nearly 41

percent of proximal tumors are CIMP+ while only 13 percent of distal and 12 percent of rectal tumors are CIMP+. Among the methylation markers proximal tumors are more likely to have *MLH1* methylation (over 17 percent) whereas this marker is seldom methylated in either distal colon or rectal tumors (3 percent and 1 percent, respectively). The differences between proximal and distal colon tumors and similarities between distal colon and rectal tumors may have potential implications for epidemiologic and molecular association studies. Distinguishing site-specific associations between proximal and distal colon tumors may be important as are distinguishing between colon and rectal cancer.

Our study has limitations. The genetic markers evaluated, although identical for colon and rectal tumors were based on markers available when the colon samples were analyzed in the mid 1990s. While new markers might provide additional information, the existing set of markers is still believed to be relevant to colorectal cancer. Because of limitations in cost-efficient manner to analyze the *APC* gene, we have not included it in our set of tumor markers. Likewise, we have sequenced the hot spots for the p53 and Ki-*ras* genes, which could result in missed mutations. However, we believe that our sequencing information provides additional data beyond that obtained from immunohistochemistry alone [40]. Although we believe that our study adds to the literature on pathogenesis of colorectal cancer, we also believe that more work is needed to better understand these pathways.

In summary, our data suggest mutational pathways that are different for proximal *vs* distal and rectal cancers. Given similarities in mutational status of distal colon and rectal cancer, it is possible that distal colon and rectal cancer share more molecular and potentially etiologic similarities than do proximal and distal colon tumors. Additional work to capture the associations between colorectal cancer tumor site and specific mutational spectra is currently being undertaken and will provide further insight into the etiology of colorectal cancer.

Acknowledgments

Acknowledgements: We would like to acknowledge the contributions of Leslie Palmer, and Judy Morse to the data collection and management efforts of this study and to Erica Wolff and Michael Hoffman for genotyping, and the core facility of the University of Utah Health Sciences Center for sequencing. We also acknowledge the support of the Biostatistics Core, NCI Cancer Center Support Grant P30CA042014, Huntsman Cancer Institute/Huntsman Cancer Foundation and support of the Data Survey and Management Core .of the Huntsman Cancer Foundation. The Institutional Review Board of all collaborating parties have reviewed and approved this project.

Grant support: This study was funded by CA48998 and CA61757 to Dr. Slattery. This research was supported by the Utah Cancer Registry, which is funded by Contract #N01-PC-67000 from the National Cancer Institute, with additional support from the State of Utah Department of Health and the University of Utah, the Northern California Cancer Registry, and the Sacramento Tumor Registry. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official view of the National Cancer Institute.

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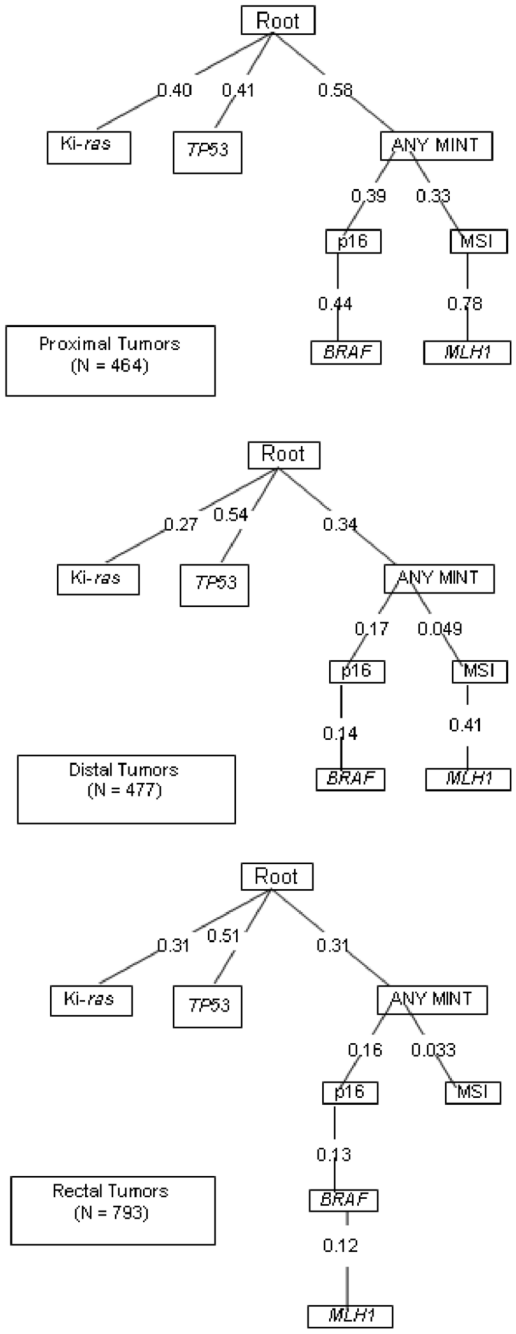

Figure 1.
Oncogenetic Tree Analysis of probability of various tumor mutations in proximal, distal, and rectal tumors.

Table 1

Comparison of somatic mutations between proximal and distal colon and rectal tumors

Mutation or Alteration Status[a]	Proximal		Distal		Rectal		*p*-value[b]
	N	(percent)	N	(percent)	N	(percent)	
Cases with p53 status	725	(100.0)	698	(100.0)	954	(100.0)	
No p53 mutation	437	(60.3)	342	(49.0)	509	(53.4)	
p53 mutation	288	(39.7)	356	(51.0)	445	(46.6)	0.01
Cases with Ki-ras status	702	(100.0)	674	(100.0)	1008	(100.0)	
No Ki-ras mutation	449	(64.0)	493	(73.1)	701	(69.5)	
Ki-ras mutation	253	(36.0)	181	(26.9)	307	(30.5)	0.03
Cases with CIMP status	607	(100.0)	595	(100.0)	889	(100.0)	
CIMP-	359	(59.1)	518	(87.1)	783	(88.1)	
CIMP+	248	(40.9)	77	(12.9)	106	(11.9)	<0.0001
Cases with MSI status	733	(100.0)	713	(100.0)	1007	(100.0)	
MSI-	559	(76.3)	686	(96.2)	985	(97.8)	
MSI+	174	(23.7)	27	(3.8)	22	(2.2)	<0.0001
Cases with BRAF status	572	(100.0)	576	(100.0)	986	(100.0)	
BRAF-	485	(84.8)	558	(96.9)	959	(97.3)	
BRAF+	87	(15.2)	18	(3.1)	27	(2.7)	<0.0001
Total cases (non-missing)[c]	501	(100.0)	502	(100.0)	858	(100.0)	
No alterations[d]	62	(12.4)	119	(23.7)	236	(27.5)	
One alteration	176	(35.1)	279	(55.6)	440	(51.3)	
Two or more alterations	263	(52.5)	104	(20.7)	182	(21.2)	<0.0001

[a] p53: positive for one or more p53 mutations in exons 5-8; Ki-ras: positive for one or more Ki-ras mutations in codons 12 and 13; MSI+: BAT26 unstable; otherwise, TGFβRII unstable if BAT26 is ambiguous or missing; CIMP+: 2 of 5 markers methylated; BRAF+: BRAF V600E mutation.

[b] Mantel-Haenszel chi-squared test, compares proximal, distal and rectal tumor site.

[c] Case subjects with status available for all alterations shown.

[d] p53 and Ki-ras negative, MSI-, and CIMP- (0 or 1 markers methylated).

Table 2

Comparison of *p53* mutations in somatic mutation with proximal and distal colon and rectal tumors

	Proximal		Distal		Rectal		
	N	(percent)	N	(percent)	N	(percent)	*p*-value[a]
Cases with p53 status[b]	725	(100.0)	698	(100.0)	954	(100.0)	
p53 negative	437	(60.3)	342	(49.0)	509	(53.4)	
p53 positive	288	(39.7)	356	(51.0)	445	(46.7)	0.01
Total p53 mutations[c]	303	(100.0)	378	(100.0)	472	(100.0)	
Missense	232	(76.6)	283	(74.9)	368	(78.0)	
Stop codon	24	(7.9)	41	(10.9)	51	(10.8)	
Frameshift	32	(10.6)	29	(7.7)	33	(7.0)	
In frame ins./del.	8	(2.6)	12	(3.2)	10	(2.1)	
Splice site	7	(2.3)	13	(3.4)	10	(2.1)	0.48
Transitions	214	(70.6)	279	(73.8)	339	(71.8)	
Transversions	48	(15.8)	57	(15.1)	88	(18.7)	
Inactivating	41	(13.5)	42	(11.1)	45	(9.5)	0.07
Non-CpG Island	158	(52.2)	192	(50.8)	247	(52.3)	
CpG Island	145	(47.9)	186	(49.2)	225	(47.7)	0.91
Non-hot spot	192	(63.4)	224	(59.3)	314	(66.5)	
R175	19	(6.3)	28	(7.4)	43	(9.1)	
G245	8	(2.6)	16	(4.2)	21	(4.5)	
R248	30	(9.9)	44	(11.6)	45	(9.5)	
R273	36	(11.9)	51	(13.5)	35	(7.4)	
R282	18	(5.9)	15	(4.0)	14	(3.0)	0.10
p53 Location							
Helix	72	(23.8)	91	(24.1)	73	(15.5)	
L3 loop	60	(19.8)	84	(22.2)	113	(23.9)	
L2 loop	42	(13.9)	63	(16.7)	102	(21.6)	
β sandwich	47	(15.5)	39	(10.3)	65	(13.8)	
Loop-sheet	20	(6.6)	27	(7.1)	27	(5.7)	
Other	62	(20.5)	74	(19.6)	92	(19.5)	0.24

	Proximal		Distal		Rectal		
	N	(percent)	N	(percent)	N	(percent)	*p*-value[a]

[a] Mantel-Haenszel chi-squared test, compares differences for proximal and distal colon and rectal.

[b] Negative (no mutations) or positive for one or more p53 mutations in exons 5-8.

Table 3

Comparison of Ki-ras mutation between proximal and distal colon and rectal tumors

	Proximal		Distal		Rectal		
	N	(percent)	N	(percent)	N	(percent)	*p*-value[a]
Cases with Ki-ras Status[b]	702	(100.0)	674	(100.0)	1008	(100.0)	
Ki-ras negative	449	(64.0)	493	(73.2)	701	(69.5)	
Ki-ras positive	253	(36.0)	181	(26.9)	307	(30.5)	0.03
Total Ki-ras mutations[c]	255	(100.0)	183	(100.0)	307	(100.0)	
Codon 12	203	(79.6)	137	(74.9)	241	(78.5)	
Codon 13	52	(20.4)	46	(25.1)	66	(21.5)	0.79
Transitions	165	(64.7)	109	(59.6)	193	(62.9)	
Transversions	90	(35.3)	74	(40.4)	114	(37.1)	0.69
Codon 12 G>A	115	(45.1)	63	(34.4)	133	(43.3)	
Codon 12 G>T	76	(29.8)	64	(35.0)	84	(27.4)	
Codon 13 G>A	50	(19.6)	46	(25.1)	60	(19.5)	
Non-hot spot	14	(5.5)	10	(5.5)	30	(9.8)	0.30

[a] Mantel-Haenszel chi-squared test, compares differences between, proximal, distal, and rectal tumors

[b] Negative (no mutations) or positive for one or more Ki-ras mutations in codons 12 and 13.

[c] Total of mutations by type may be greater than total Ki-ras+ cases because of individuals with multiple mutations.

Table 4

Comparison of CIMP, MSI, and BRAF in proximal and distal colon and rectal cases

	Proximal		Distal		Rectal		
	N	(percent)	N	(percent)	N	(percent)	p-value[a]
Cases with CIMP status[b]	607	(100.0)	595	(100.0)	889	(100.0)	
CIMP-	359	(59.1)	518	(87.1)	783	(88.1)	
CIMP+	248	(40.9)	77	(12.9)	106	(11.9)	<0.0001
Cases with MLH1 status	587	(100.0)	577	(100.0)	909	(100.0)	
MLH1-	485	(82.6)	560	(97.1)	899	(98.9)	
MLH1 +	102	(17.4)	17	(3.0)	10	(1.1)	<0.0001
Cases with p16 status	618	(100.0)	606	(100.0)	946	(100.0)	
p16-	471	(76.2)	558	(92.1)	868	(91.8)	
p16+	147	(23.8)	48	(7.9)	78	(8.3)	<0.0001
Cases with MINT1 status	608	(100.0)	601	(100.0)	920	(100.0)	
MINT1-	442	(72.7)	561	(93.3)	884	(96.1)	
MINT1 +	166	(27.3)	40	(6.7)	36	(3.9)	<0.0001
Cases with MINT2 status	613	(100.0)	602	(100.0)	906	(100.0)	
MINT2-	380	(62.0)	482	(80.1)	727	(80.2)	
MINT2+	233	(38.0)	120	(19.9)	179	(19.8)	<0.0001
Cases with MINT31 status	590	(100.0)	599	(100.0)	913	(100.0)	
MINT31-	319	(54.1)	489	(81.6)	740	(81.1)	
MINT31+	271	(45.9)	110	(18.4)	173	(19.0)	<0.0001
Number of markers methylated							
0	243	(40.0)	383	(64.4)	594	(66.8)	
1	116	(19.1)	135	(22.7)	189	(21.3)	
2	87	(14.3)	48	(8.1)	67	(7.5)	
3	61	(10.1)	17	(2.9)	28	(3.2)	
4	62	(10.2)	10	(1.7)	10	(1.1)	
5	38	(6.3)	2	(0.3)	1	(0.1)	<0.0001
CIMP- cases with MSI status	345	(100.0)	501	(100.0)	779	(100.0)	

	Proximal		Distal		Rectal		
	N	(percent)	N	(percent)	N	(percent)	p-value[a]
MSI-	319	(92.5)	485	(96.8)	766	(98.3)	
MSI+	26	(7.5)	16	(3.2)	13	(1.7)	<0.0001
CIMP+ cases with MSI status	243	(100.0)	74	(100.0)	105	(100.0)	
MSI-	138	(56.8)	67	(90.5)	101	(96.2)	
MSI+	105	(43.2)	7	(9.5)	4	(3.8)	<0.0001
CIMP- cases with BRAF status	328	(100.0)	485	(100.0)	768	(100.0)	
BRAF-	319	(97.3)	482	(99.4)	760	(99.0)	
BRAF+	9	(2.7)	3	(0.6)	8	(1.0)	0.06
CIMP+ cases with BRAF status	231	(100.0)	73	(100.0)	102	(100.0)	
BRAF-	155	(67.1)	58	(79.5)	84	(82.4)	
BRAF+	76	(32.9)	15	(20.5)	18	(17.7)	<0.01

[a] Mantel-Haenszel chi-squared test, compares differences by proximal, distal, and rectal tumors.

[b] CIMP-: 0 or 1 of 5 markers methylated; CIMP+: 2 or more of 5 markers methylated.