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The *MLH1* -93 G>A Promoter Polymorphism and Genetic and Epigenetic Alterations in Colon Cancer

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Abstract

The *MLH1* -93 G>A promoter polymorphism has been reported to be associated with an increased risk of microsatellite unstable colorectal cancer. Other than microsatellite instability, however, the genetic and most epigenetic changes of tumors associated with this polymorphism have not been studied. We evaluated associations between the -93 G>A polymorphism and CpG island methylator phenotype (CIMP), *BRAF* V600E mutations, and *MLH1* methylation in tumors from a sample of 1,211 individuals with colon cancer and 1,968 controls from Utah, Northern California and Minnesota. The -93 G>A polymorphism was determined by the five prime nuclease assay. CIMP was determined previously by methylation-specific PCR of CpG islands in *MLH1*, methylated in tumors (MINT)1, MINT2, MINT31 and *CDKN2A* (*p16*). The *BRAF* V600E mutation was determined by sequencing exon 15. The *MLH1* -93 G>A promoter polymorphism was associated with CIMP (odds ratio (OR) 3.44, 95% confidence interval (CI) 1.85, 6.42), *MLH1* methylation (OR 4.16, 95%CI 2.20, 7.86), *BRAF* mutations (OR 4.26, 95%CI 1.83, 9.91) and older age at diagnosis (OR 3.65, 95%CI 2.08, 6.39) in microsatellite unstable tumors. These associations were not observed in stable tumors. Increased age at diagnosis and tumor characteristics of microsatellite unstable tumors associated with *MLH1* -93 G>A suggests the polymorphism is acting at a relatively late stage of colorectal carcinogenesis to drive CIMP+ tumors down the microsatellite instability pathway.

INTRODUCTION

Microsatellite instability (MSI), the expansion or contraction of short nucleotide repeats, occurs in 10–15% of colorectal cancers (Smyrk, 1994). The more common sporadic form of MSI is usually secondary to acquired hypermethylation of the promoter of one of the mismatch repair genes, *MLH1* (Cunningham et al., 1998). This also is commonly associated with widespread methylation of CpG islands, or the CpG island methylator phenotype (CIMP), and activating mutations in the oncogene *BRAF* (Samowitz et al., 2005). Hereditary non-polyposis colorectal cancer (HNPCC or Lynch syndrome), the inherited form of tumoral MSI, is due to germline mutations in one of the mismatch repair genes, either *MLH1*, *MSH2*, *MSH6*, or *PMS2*. HNPCC-associated tumors typically do not have *MLH1* methylation, *BRAF* mutations or CIMP (Samowitz, 2007).

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A recent study reported that a polymorphism in the promoter of *MLH1*, the -93 G>A (rs1800734), was associated with an increased risk of microsatellite unstable colorectal cancers in two populations, but no change in the risk of stable tumors was observed (Raptis et al., 2007). The polymorphism was also associated with a family history of colorectal cancer. It thus appeared to be acting much like a germline mutation in *MLH1*, with the exception that it did not appear to be as penetrant as typical *MLH1* mutations and an association with younger age of onset was not seen (Raptis et al., 2007). Also, only about one-third of tumors associated with the homozygous -93 AA genotype exhibited MSI, while nearly all HNPCC-associated colorectal cancers are unstable (Aaltonen et al., 1994).

An evaluation of the -93 G>A polymorphism in our study population has confirmed the association with an increased risk of microsatellite unstable tumors (Hubner and Houlston, 2007), and another study showed an association with *MLH1* methylation (Chen et al., 2007). Other genetic and epigenetic changes of the cancers associated with the -93 G>A polymorphism have not been evaluated, however. In particular, it would be of considerable interest to see whether the associated tumors behave more like sporadic unstable cancers – that is, are associated with CIMP and *BRAF* mutations in addition to *MLH1* methylation– or more like HNPCC-associated tumors, which are typically not associated with these genetic and epigenetic changes. It should be noted that *MLH1* methylation is not always associated with other molecular features typical of sporadic microsatellite unstable tumors; rare individuals have been described with another germline promoter alteration, namely *MLH1* methylation (Hitchins et al., 2007), and their associated tumors do not harbor *BRAF* mutations (N. Hawkins, personal communication). We therefore evaluated the *MLH1* -93 G>A polymorphism and associated clinical and tumor characteristics in a sample of 1,211 individuals with colon cancer and 1,968 controls from Utah, Northern California and Minnesota.

MATERIALS AND METHODS

Study Population

Study participants were predominantly non-Hispanic white (91% of cases and 93% of controls), Hispanic, or black and were from either the Kaiser Permanente Medical Care Program (KPMCP) of Northern California, an eight county area in Utah (Davis, Salt Lake, Utah, Weber, Wasatch, Tooele, Morgan, and Summit counties), or the Twin Cities Metropolitan area in Minnesota. Eligibility criteria for cases included diagnosis with first-primary incident colon cancer (ICD-O 2nd edition codes 18.0, 18.2 to 18.9) between October 1, 1991 and September 30, 1994, between 30 and 79 years of age at time of diagnosis, and mentally competent to complete the interview. Cases with adenocarcinoma or carcinoma of the rectosigmoid junction or rectum (defined as the first 15 cm from the anal opening), or with known familial adenomatous polyposis, ulcerative colitis, or Crohn's disease were not eligible. Of all cases asked to participate, 75.6% cooperated.

Controls were selected from eligibility lists for KPMCP and random-digit-dialing, driver's license lists for Minnesota, and random-digit-dialing, driver's license lists, or Health Care Finance Administration (HCFA) lists for Utah, using the same eligibility criteria as was used for cases. These methods have been described in detail (Slattery et al., 1997). Of all controls contacted, 63.7% participated. Cases or controls who reported a history of colorectal cancer in a first degree relative were categorized as having a positive family history.

MLH1 -93 G>A Polymorphism

The *MLH1* -93 G>A promoter polymorphism was determined as described previously (Yu et al., 2006). Briefly, PCR amplification of the genomic DNA in this region was followed by

allelic discrimination using the 5' nuclease assay on a 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Validation of the 5' nuclease assay was conducted among 92 individuals by comparing genotypes generated by the 5' nuclease assay to those detected by RFLP or sequencing. There were no discrepancies between assays. Positive and negative controls were included on each plate. For additional quality control, genotyping of 94 randomly selected samples was repeated with the same 5' nuclease assay. There were no discrepancies between runs. The genotyping failure rate for this polymorphism was <0.01.

Microsatellite Instability (MSI)

The MSI status of the tumors had been determined in a previous study (Samowitz et al., 2001b). Of 1,608 cases with study data and *MLH1* -93G>A genotyped from blood DNA, we obtained tumor blocks from 1,250 subjects (78%); adequate DNA for instability results was available for 1,211 tumors (97%). This study preceded the development of the Bethesda consensus panel; the MSI markers used were BAT-26 (a mononucleotide repeats which by itself is a very good measure of generalized instability), *TGFBR2* (a coding mononucleotide repeat which is unstable in most colorectal cancers with microsatellite instability) and a panel of 10 tetranucleotide repeats which show a high correlation with the Bethesda consensus panel and BAT-26 (Samowitz et al., 2001c). A hierarchical approach was then used for microsatellite instability determination. A majority of tumors (95%) were classified (either stable or unstable) for BAT-26. Sixty-one tumors (which did not have a result for BAT-26) were classified using *TGFBR2* and four tumors (with no result for either BAT-26 or *TGFBR2*) were classified using the panel of 10 tetranucleotide repeats; if 30% or more of the 10 tetranucleotide repeats were unstable, the tumor was classified as unstable.

BRAF V600E Mutation Detection

The *BRAF* V600E mutation status of the tumors was determined in a previous study (Samowitz et al., 2005). Briefly, exon 15 of *BRAF* was PCR-amplified from DNA previously extracted from tumors microdissected from formalin-fixed, paraffin-embedded blocks (Spirio et al., 1998) using the forward primer 5'-TCA TAA TGC TTG CTC TGA TAG GA-3' and the reverse primer 5'-CTT TCT AGT AAC TCA GCA GC-3'. Amplifications were carried out using AmpliTaq Gold and a PCR profile consisting of a 9 min. initial denaturation at 95°C followed by 35 cycles of 20 s at 95°C, 20 s at 60°C, and 30 s at 72°C with a 5 min. final extension at 72°C. Mutations were verified by sequencing in both directions. Sufficient DNA was available for *BRAF* V600E determination for 1,009 tumors.

CpG Island Methylator Phenotype (CIMP)

The CIMP status of the tumors was determined in a previous study (Samowitz et al., 2005). Briefly, sodium bisulfite modification was performed on DNA extracted from tumors microdissected from formalin-fixed, paraffin-embedded blocks. Methylation-specific PCR was then performed as described previously for the following CpG islands: MINT1, MINT2, MINT31, *CDKN2A* (*p16*), and *MLH1*. The criterion for CIMP+ was methylation of two or more of these CpG islands. CIMP- was defined as less than two of five markers methylated. The use of established assays, selection of genetic loci, and criterion for CIMP+ or CIMP- was based on the pioneering work of other groups that previously defined the CIMP phenotype (Frazier et al., 2003; Park et al., 2003). The primers used for *MLH1* methylation were located ~170 and 270 base pairs 5' of the start codon. This corresponds to the region previously shown to be correlated with the absence of gene expression (Deng et al., 1999). Sufficient DNA was available for CIMP determination for 1,001 tumors.

Approximately 15–20% of cases for which microsatellite instability status was determined previously did not have a subsequent result for CIMP, *MLH1* methylation, or *BRAF* due to assay failure or insufficient DNA.

Statistical Analysis

A χ^2 test was used to determine if the observed genotype frequencies differed from Hardy-Weinberg equilibrium among the 1,968 study controls and among the 1,211 study cases. Odds ratios and 95% confidence intervals were calculated from unconditional multivariate logistic regression models using SAS® (release 9.1.3) to estimate the risk of colon cancer associated with *MLH1* -93G>A stratified by MSI status. Adjustment for potential confounding variables, where appropriate, included age at diagnosis or selection, sex, race/ethnicity, study center, and smoking amount (number of cigarettes smoked per day on a regular basis). These variables were included in the multivariate adjustment as we previously reported that MSI, CIMP, and *BRAF* status were associated with age, sex, and smoking (Samowitz et al., 2005, 2006; Slattery et al., 2000, 2001); however, results did not significantly differ when they were excluded from the analyses. For risk of CIMP, *MLH1* methylation, or *BRAF* mutation in colon cancers associated with *MLH1* -93G>A, data were analyzed using polytomous logistic regression models comparing cases with and without CIMP, *MLH1* methylation, or *BRAF* mutation to controls. A case-case comparison comparing cases with unstable tumors to cases with stable tumors was also performed. Assessment of interactions between genotypes and tumor characteristics, age, and family history were based on a multiplicative scale. Effect modification was determined by evaluating the improvement in fit (difference in -2 log likelihood values) of a model that included a multiplicative interaction term compared to a restricted model with no interaction term.

RESULTS

Characteristics of colon cancer cases and controls are shown in Table 1. The -93 G>A genotypes were in Hardy-Weinberg equilibrium both for the 1,968 controls and for the 1,211 cases, analyzed separately (data not shown). A relationship between the *MLH1* -93 G>A promoter polymorphism and an increased risk of microsatellite unstable cancer has already been shown in this population (Hubner and Houlston, 2007).

The relationships between this polymorphism and CIMP, *MLH1* methylation and the *BRAF* V600E mutation in tumors are shown in Table 2. The G>A polymorphism was associated with a significantly increased risk of microsatellite unstable tumors with CIMP+, *MLH1* methylation, and *BRAF* mutations as compared to controls (AA genotype: OR 3.44, 95%CI 1.85, 6.42; OR 4.16, 95%CI 2.20, 7.86; and OR 4.26, 95%CI 1.83, 9.91, respectively; p trends <0.01). An increased risk of these genetic and epigenetic alterations was not seen in microsatellite stable tumors. Indeed, there was a trend towards a decreased risk of microsatellite stable CIMP+ cancers (OR for the AA genotype 0.53, 95%CI 0.21, 1.33; p trend=0.10). The G>A polymorphism was also associated with an increased risk of these genetic and epigenetic changes in a case-case comparison of microsatellite unstable vs. stable cancers, with significant p-values for heterogeneity for CIMP+ and *BRAF* V600E mutations (0.002 and 0.02, respectively). The *MLH1* methylation p-value was not significant, but this comparison involved very small numbers of tumors. The data indicate a possible contribution by the heterozygous GA genotype, especially with respect to *BRAF* mutations for microsatellite unstable cancers (OR 1.72, 95% C.I. 0.97, 3.04), and in the case-case comparison.

The interaction between age and the -93 G>A polymorphism in modifying colon cancer risk is shown in Table 3. The polymorphism only increased the risk of microsatellite unstable

cancers in individuals 60 years of age or older (OR for the AA genotype 3.65, 95%CI 2.08, 6.39, p interaction =0.02). The polymorphism had no effect on the risk of an unstable tumor in younger individuals, or on the risk of a microsatellite stable cancer in individuals of any age. No effect was seen by the GA heterozygote in unstable tumors.

The relationship between the -93 G>A polymorphism, a family history of colorectal cancer, and microsatellite instability was not statistically significant (Table 4, p interaction= 0.18), although the odds ratio for the AA genotype in individuals with unstable tumors and a positive family history (OR 12.45, 95% C.I. 4.85, 32.0) was higher than that for a negative family history (OR 1.96, 95% C.I. 1.04, 3.69). No effect was seen for the GA heterozygote in unstable tumors.

DISCUSSION

Previous studies, including our own, have shown an association between the -93 G>A *MLH1* promoter polymorphism and an increased risk of microsatellite unstable colon cancer. The molecular alterations in these unstable tumors have not been completely characterized, however. In this study we show that in microsatellite unstable cancers this polymorphism was associated with *MLH1* methylation (as previously described by Chen et al. (2007), CIMP, and *BRAF* V600E mutations. In addition, and in contrast to the previous study (Raptis et al., 2007) which found no relationship with age, in our population the -93 G>A polymorphism was associated with increased age at diagnosis in individuals with microsatellite unstable tumors. These features, including increased age, are typically associated with sporadic, rather than HNPCC-associated, microsatellite unstable colorectal cancers. In the current study, the increased risk of the -93 AA genotype in unstable tumors was seen in those both with and without a family history of colorectal cancer, although there was a suggestion of a stronger association in those with a positive family history. The previous study reported an association of the -93 G>A polymorphism with family history but did not separate stable from unstable cancers in their analysis (Raptis et al., 2007). None of these associations were seen in microsatellite stable cancers.

We thus have evidence of a cancer-predisposing germline change in a mismatch repair gene which is associated with clinical and pathological features more typical of sporadic microsatellite unstable tumors. New data regarding oncogenic pathways in colon cancer may offer some explanation for this apparent contradiction, especially as it relates to the stage of carcinogenesis which might be affected by the -93 G>A polymorphism. First of all, there is some evidence that CIMP and *BRAF* mutations may precede *MLH1* methylation and microsatellite instability in sporadic colorectal cancers. CIMP and *BRAF* mutations are commonly seen in a subset of hyperplastic polyps of the colon (O'Brien et al., 2006), and it has been theorized that these lesions may be the precursors of CIMP+ unstable cancers (Kambara et al., 2004). These polyps do not show evidence of loss of expression of *MLH1* (Sheridan et al., 2006) or MSI (O'Brien et al., 2006); loss of expression is typically not seen in these polyps until a later stage, in which adenomatous areas develop (Sheridan et al., 2006), and high degrees of MSI are typically seen only at the cancerous stage of sporadic colon carcinogenesis or in adenomatous areas adjacent to cancers (O'Brien et al., 2006). A sequence of events in sporadic carcinogenesis in which CIMP and *BRAF* mutations are followed by loss of *MLH1* expression (presumably due to methylation) and then microsatellite instability thus seems reasonable.

There is evidence for similarities between CIMP+ microsatellite stable and unstable tumors. Both types of tumors share associations with the V600E *BRAF* mutation, proximal location, older age, and certain histologic features (Chirieac et al., 2005; Samowitz et al., 2005). Also, in a recent study we found that smoking predisposed to CIMP and *BRAF* mutations in both

microsatellite stable and unstable cancers (Samowitz et al., 2006). Although alternative explanations are possible, a potential explanation is that CIMP+ microsatellite stable and unstable tumors share a common precursor. Our recent study of *APC* mutations in colon cancer provides some support for this notion, as we noted an inverse relationship between *APC* mutations and *BRAF* mutations in microsatellite stable carcinomas (Samowitz et al., 2007). Since *APC* mutations are thought to be the initial mutation in adenomatous polyps (Powell et al., 1992), and nearly all *BRAF*-mutated tumors are CIMP+ (Samowitz et al., 2005), this suggests the possibility of a non-adenomatous precursor for CIMP+ microsatellite stable cancers, perhaps the same CIMP+, *BRAF*-mutated hyperplastic polyps which are thought to give rise to microsatellite unstable carcinomas. An association between smoking and hyperplastic polyps has also been reported (Morimoto et al., 2002; Potter et al., 1999). All of this is consistent with a carcinogenic model in which the development of CIMP+ –with its attendant associations– is either followed by *MLH1* methylation, in which case a microsatellite unstable tumor develops– or is not, in which case a CIMP+ microsatellite stable cancer develops. This is consistent with the model originally proposed for CIMP in microsatellite stable and unstable tumors, although that model envisaged adenomatous polyps as the common precursor (Toyota et al., 1999).

Given this model, the -93 G>A polymorphism may be acting during the CIMP+ stage of tumorigenesis to increase the likelihood of *MLH1* methylation, as shown in Figure 1. How it might do this is not clear, but the -93 is located in a CpG island and near other CpG islands of the promoter (Raptis et al., 2007). Whatever the mechanism, a polymorphism which drives CIMP+ tumors down the MSI pathway would explain most of the results seen with this polymorphism. This includes, obviously, the relationship to *MLH1* methylation and MSI, but also the association with CIMP+ and factors associated with CIMP, such as *BRAF* mutations and increased age. This is consistent with the markedly increased risk of a microsatellite unstable, CIMP+ cancer as compared to a microsatellite stable, CIMP+ cancer associated with the AA polymorphism (OR 6.49, 95% CI 2.16, 19.5). Such a model would predict a relative deficit of CIMP+ stable tumors associated with this polymorphism, since the polymorphism favors *MLH1* methylation and subsequent MSI development in CIMP+ lesions. Indeed, a suggestion of an inverse relationship between -93G>A and CIMP+ was seen in stable cancers (Table 2). Finally, the relative lack of penetrance of the *MLH1* -93 G>A polymorphism as compared to more typical germline mismatch repair gene mutations could be partly due to this polymorphism only exerting its effect in the relatively uncommon context of a CIMP+ background.

Interestingly, there is no evidence in this population of a relationship between the -93 G>A polymorphism, smoking and MSI (data not shown). As noted above, we have previously described associations between cigarette smoking and MSI (Slattery et al., 2000), CIMP and *BRAF* mutations (Samowitz et al., 2006). The relationship to CIMP and *BRAF* was independent of MSI, suggesting that smoking was related to instability indirectly through its association with methylation; indeed, compounds in cigarette smoke activate the aromatic hydrocarbon receptor which can lead to DNA methylation (Dertinger et al., 1998; Ray, 2004). Risk factors other than cigarette smoking have not been identified for CIMP and *BRAF*. Our current results suggest that the -93 G>A polymorphism is associated with an alternative pathway to CIMP/*BRAF*/MSI. Also of interest, there are rare individuals with Lynch/HNPCC due to germline methylation of *MLH1* or *MSH2* (Hitchins et al., 2007). Their associated tumors do not have *BRAF* mutations (N Hawkins, personal communication), and so this epigenetic change appears to be acting more like a classic HNPCC germline mutation than the -93 polymorphism.

This model is not meant to explain all microsatellite unstable colon cancers. We and others have noted a subset of apparently sporadic microsatellite unstable tumors without CIMP

and/or *MLH1* methylation (Ogino et al., 2007; Oliveira et al., 2004; Samowitz et al., 2005). Interestingly, we have also identified an association between alcohol use and CIMP negative, microsatellite high colon cancers (Slattery et al., 2007). Our model also does not explain the rare examples (less than 2%) of microsatellite stable tumors with *MLH1* methylation.

A recent correspondence raised the possibility of linkage disequilibrium between the -93 G/A polymorphism and *MLH1* germline mutations as being the actual cause of the association of the polymorphism with MSI (Hubner and Houlston, 2007). Tumor characteristics associated with the polymorphism in the current study argues against this, as one would not expect to see *MLH1* methylation, CIMP+ and *BRAF* mutations in tumors associated with germline mismatch repair gene mutations. An association between the -93 G>A polymorphism and mismatch repair gene mutations was not seen in the previously studied population (Hubner and Houlston, 2007). It is possible the G>A polymorphism is in linkage disequilibrium with another causal polymorphism in the relationship with MSI. There is linkage disequilibrium with the *MLH1* I219V polymorphism, but the -93 AA genotype only occurs with the common codon 219 isoleucine, and no association was seen between *MLH1* I219V and MSI (data not shown).

We did not attempt to exclude individuals with HNPCC from this study. Indeed, seven known mutation carriers were included: four had genotype GG and three had genotype GA. This translates to approximately 4% of individuals with microsatellite unstable tumors, very similar to the approximately 5% estimate from our population-based sample (which included many of these patients) (Samowitz et al., 2001a). Including this small percentage would not be expected to have an impact on our analyses. It is important to recognize, however, that our original question was whether the -93 G>A polymorphism-associated unstable tumors were more like sporadic unstable tumors or more like those associated with HNPCC. In Tables 2 and 3 we are actually performing a stricter comparison. Since the great preponderance of unstable tumors are sporadic, we are showing that even compared to sporadic unstable tumors the -93-associated cancers were more likely to exhibit features associated with sporadic tumors; that is, be CIMP+, *MLH1* methylated, *BRAF* mutated and occur in older individuals. With respect to Table 4, inclusion of HNPCC-associated tumors would only affect the familiarity comparison if these were in linkage disequilibrium with the AA genotype, which does not appear to be the case in our known gene carriers or in a previous study (Hubner and Houlston, 2007).

A limitation of studying DNA methylation in colon tumors is that a gold standard for CIMP classification is lacking. There is some evidence for three classes of CIMP, namely CIMP negative, CIMP low and CIMP positive (Shen et al., 2007). In such a classification CIMP high tumors are associated with *BRAF* mutations and CIMP low tumors are associated with *KRAS2* mutations (Ogino et al., 2006; Shen et al., 2007). The classic CIMP panel utilized in the current study probably combines both CIMP low and CIMP high, since CIMP as defined by our panel is associated with both *KRAS2* and *BRAF* mutations, two generally mutually exclusive events. Another panel proposed by Weisenberger et al. (2006) leads to an extremely strong association between CIMP positive and *BRAF* mutations; in this classification CIMP negative probably includes some CIMP low cases. The biologic significance and clinical utility of CIMP low is somewhat uncertain at this time, although we did find an association between cigarette smoking and both CIMP positive/*BRAF* mutated and CIMP positive/*BRAF* wild type tumors (Samowitz et al., 2006), roughly analogous to CIMP high and low, respectively. At any rate, while our CIMP definition may be somewhat more inclusive than that derived from other CIMP panels, it does identify many relationships seen by other groups using different methylation panels, including associations with *BRAF* mutations, right-sided location, microsatellite instability, older age and increased

stage (Samowitz et al., 2005). Also, the similarity of these -93 G>A associated unstable tumors to sporadic unstable tumors (and dissimilarity to Lynch/HNPCC-associated cancers) is also strongly supported by the associations with *BRAF* mutations and *MLH1* methylation.

In summary, we have identified associations between the -93 G>A polymorphism and *MLH1* methylation, CIMP+ and *BRAF* V600E mutations in microsatellite unstable colonic cancers, as well as increased age at diagnosis. These features suggest that the polymorphism is acting at a relatively late stage of colorectal carcinogenesis to drive CIMP+ tumors down the MSI pathway.

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Figure 1.

It is hypothesized that the *MLH1* -93 AA genotype predisposes a CIMP+ precursor to undergo *MLH1* methylation with the subsequent development of microsatellite instability.

TABLE 1

Description of Cases and Controls

Characteristic	Controls		Cases	
	Number	(%)	Number	(%)
Total	1968	(100.0)	1211	(100.0)
Age, y				
30–39	41	(2.1)	15	(1.2)
40–49	131	(6.7)	78	(6.4)
50–59	329	(16.7)	218	(18.0)
60–69	672	(34.1)	427	(35.3)
70–79	795	(40.4)	473	(39.1)
Center				
Northern California	816	(41.5)	597	(49.3)
Minnesota	805	(40.9)	398	(32.9)
Utah	347	(17.6)	216	(17.8)
Sex				
Male	1042	(52.9)	662	(54.7)
Female	926	(47.1)	549	(45.3)
Race/ethnicity				
White, non-Hispanic	1833	(93.1)	1096	(90.5)
White, Hispanic	79	(4.0)	52	(4.3)
Black	54	(2.7)	60	(5.0)
Other	2	(0.1)	3	(0.2)
Family history, 1st degree				
None	1785	(90.7)	1020	(84.2)
1 relative	166	(8.4)	159	(13.1)
2 relatives	13	(0.7)	28	(2.3)
>2 relatives	4	(0.2)	4	(0.3)
<i>MLH1</i> -93G>A				
GG	1173	(59.6)	724	(59.8)
GA	689	(35.0)	414	(34.2)
AA	106	(5.4)	73	(6.0)

Characteristic	Controls		Cases	
	Number	(%)	Number	(%)
Site of tumor				
Proximal			622	(51.4)
Distal			564	(46.6)
Colon, not specified			25	(2.1)
AJCC stage				
1			336	(27.7)
2			392	(32.4)
3			364	(30.1)
4			103	(8.5)
Unknown			16	(1.3)
MSI status				
Stable			1018	(84.1)
Unstable			193	(15.9)
CIMP status				
CIMP– (0 or 1 marker methylated)			721	(59.5)
CIMP+ (2 or more of 5 methylated)			280	(23.1)
<i>MLH1</i> methylation				
Unmethylated			897	(74.1)
Methylated			114	(9.4)
<i>BRAF</i> V600E				
Wild type			910	(75.1)
Mutated			99	(8.2)

TABLE 2
Association of CIMP, *MLH1* Methylation, and *BRAF* V600E Mutations With *MLH1* -93G>A Genotype by MSI Status

Microsatellite stable Tumors																	Microsatellite unstable Tumors										Unstable vs. stable					
Genotype	Controls		CIMP-		95% CI		CIMP+		95% CI		CIMP-		CIMP+		95% CI		CIMP-		CIMP+		95% CI		CIMP-		CIMP+		95% CI					
	N	OR	N	OR	Lower	Upper	N	OR	Lower	Upper	N	OR	Lower	Upper	N	OR	Lower	Upper	N	OR	Lower	Upper	N	OR	Lower	Upper	N	OR	Lower	Upper		
GG	1170	399	1.00	-Ref-	-Ref-		111	1.00	-Ref-		35	1.00	-Ref-		56	1.00	-Ref-		56	1.00	-Ref-		1.00	-Ref-		1.00	-Ref-		(0.79 , 2.36)			
GA	688	237	1.02	(0.84 , 1.23)	(0.57 , 1.14)		52	0.81	(0.57 , 1.14)		12	0.58	(0.30 , 1.14)		40	1.21	(0.79 , 1.85)		40	1.21	(0.79 , 1.85)		1.37	(0.79 , 2.36)		1.37	(0.79 , 2.36)		(0.79 , 2.36)			
AA	105	32	0.89	(0.59 , 1.35)	(0.21 , 1.33)		5	0.53	(0.21 , 1.33)		2	0.62	(0.14 , 2.62)		15	3.44	(1.85 , 6.42)		15	3.44	(1.85 , 6.42)		6.49	(2.16 , 19.5)		6.49	(2.16 , 19.5)		(2.16 , 19.5)			
GG	1170	<i>MLH1</i> -U		95% CI		<i>MLH1</i> -M		95% CI		<i>MLH1</i> -U		95% CI		<i>MLH1</i> -M		95% CI		<i>MLH1</i> -U		95% CI		<i>MLH1</i> -M		95% CI		<i>MLH1</i> -U		95% CI		<i>MLH1</i> -M		
		N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	
		511	1.00	-Ref-		10	1.00	-Ref-		40	1.00	-Ref-		49	1.00	-Ref-		49	1.00	-Ref-		49	1.00	-Ref-		1.00	-Ref-		1.00	-Ref-		
		290	0.98	(0.82 , 1.16)		4	0.68	(0.21 , 2.20)		15	0.63	(0.34 , 1.15)		35	1.24	(0.79 , 1.95)		35	1.24	(0.79 , 1.95)		35	1.24	(0.79 , 1.95)		1.70	(0.45 , 6.4)		1.70	(0.45 , 6.4)		
GA	688	290	0.98	(0.82 , 1.16)	(0.21 , 2.20)		4	0.68	(0.21 , 2.20)		15	0.63	(0.34 , 1.15)		35	1.24	(0.79 , 1.95)		35	1.24	(0.79 , 1.95)		1.70	(0.45 , 6.4)		1.70	(0.45 , 6.4)		(0.45 , 6.4)			
AA	105	34	0.75	(0.50 , 1.12)	(0.16 , 10.1)		1	1.25	(0.16 , 10.1)		2	0.53	(0.12 , 2.24)		15	4.16	(2.20 , 7.86)		15	4.16	(2.20 , 7.86)		2.39	(0.24 , 23.4)		2.39	(0.24 , 23.4)		(0.24 , 23.4)			
GG	1170	<i>BRAF</i> _{WT}		95% CI		<i>BRAF</i> _{MUT}		95% CI		<i>BRAF</i> _{WT}		95% CI		<i>BRAF</i> _{MUT}		95% CI		<i>BRAF</i> _{WT}		95% CI		<i>BRAF</i> _{MUT}		95% CI		<i>BRAF</i> _{WT}		95% CI		<i>BRAF</i> _{MUT}		
		N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	N	OR	
		496	1.00	-Ref-		27	1.00	-Ref-		56	1.00	-Ref-		25	1.00	-Ref-		56	1.00	-Ref-		25	1.00	-Ref-		1.00	-Ref-		1.00	-Ref-		
		285	0.98	(0.82 , 1.17)		12	0.79	(0.40 , 1.58)		24	0.74	(0.45 , 1.21)		25	1.72	(0.97 , 3.04)		25	1.72	(0.97 , 3.04)		25	1.72	(0.97 , 3.04)		2.63	(0.96 , 7.24)		2.63	(0.96 , 7.24)		
GA	688	285	0.98	(0.82 , 1.17)	(0.40 , 1.58)		12	0.79	(0.40 , 1.58)		24	0.74	(0.45 , 1.21)		25	1.72	(0.97 , 3.04)		25	1.72	(0.97 , 3.04)		2.63	(0.96 , 7.24)		2.63	(0.96 , 7.24)		(0.96 , 7.24)			
AA	105	36	0.82	(0.55 , 1.22)	(0.21 , 3.94)		2	0.92	(0.21 , 3.94)		9	1.88	(0.90 , 3.96)		8	4.26	(1.83 , 9.91)		8	4.26	(1.83 , 9.91)		5.09	(0.81 , 31.7)		5.09	(0.81 , 31.7)		(0.81 , 31.7)			

Adjusted for age, sex, race, center, and usual smoking amount (cigarettes/day). Number of subjects varies from Table 1 due to missing data (approximately 15–20% of cases for which microsatellite instability was determined)for CIMP, *MLH1* or *BRAF* due to assay failure or insufficient DNA. CIMP- was defined as 0 or 1 of 5 CpG islands methylated; CIMP+ was defined as 2 or more of 5 CpG islands methylated (see Methods). *MLH1*-U (unmethylated), *MLH1*-M (methylated).

TABLE 3
MLH1 -93G>A Polymorphism and Interaction With Age in Colon Cancer Risk by MSI Status

Age ≥ 60												Age < 60					
Controls			All cases			95% CI			Controls			All cases			95% CI		
Genotype	N		N	OR	Lower	Upper			N			N	OR	Lower	Upper		
GG	883		530	1.00	-Ref-				287			190	1.08	(0.87	, 1.34)		0.05
GA	513		309	1.01	(0.85	, 1.21)			175			103	0.98	(0.75	, 1.29)		
AA	66		58	1.47	(1.01	, 2.13)			39			15	0.66	(0.36	, 1.21)		
Controls			Stable cases			95% CI			Controls			Stable cases			95% CI		
Genotype	N		N	OR	Lower	Upper			N			N	OR	Lower	Upper		P- Interact
GG	883		455	1.00	-Ref-				287			161	1.07	(0.85	, 1.34)		0.31
GA	513		257	0.98	(0.81	, 1.18)			175			88	0.99	(0.74	, 1.31)		
AA	66		38	1.13	(0.74	, 1.71)			39			13	0.67	(0.35	, 1.26)		
Controls			Unstable cases			95% CI			Controls			Unstable cases			95% CI		
Genotype	N		N	OR	Lower	Upper			N			N	OR	Lower	Upper		P- Interact
GG	883		75	1.00	-Ref-				287			29	1.17	(0.75	, 1.84)		0.02
GA	513		52	1.18	(0.81	, 1.72)			175			15	0.99	(0.56	, 1.78)		
AA	66		20	3.65	(2.08	, 6.39)			39			2	0.61	(0.14	, 2.58)		

Adjusted for sex, race, center and usual smoking amount. Number of subjects varies slightly from Table 1 due to missing data for one or more adjustment variables. Age <60 represents the 25th percentile in study controls and in study cases.

TABLE 4
MLH1 -93G>A Polymorphism and Interaction With Family History of Colorectal Cancer (CRC) by MSI Status

No Family History CRC										Positive Family History of CRC					
Genotype	Controls		All cases		95% CI		Controls		All cases		95% CI		Upper	P-Interact	P-Interact
	N		N	OR	Lower	Upper	N		N	OR	Lower	Upper			
GG	1059		603	1.00	-Ref-		111		117	1.90	(1.44	, 2.52)			0.64
GA	626		354	1.01	(0.85	, 1.19)	62		58	1.69	(1.16	, 2.46)			
AA	95		58	1.08	(0.77	, 1.53)	10		15	2.84	(1.26	, 6.40)			
Stable cases															
Unstable cases															
95% CI															
Genotype	N		N	OR	Lower	Upper	Controls	N		OR	Lower	Upper			
GG	1059		526	1.00	-Ref-		111		90	1.69	(1.25	, 2.29)			0.95
GA	626		298	0.97	(0.82	, 1.16)	62		47	1.56	(1.05	, 2.32)			
AA	95		45	0.96	(0.66	, 1.40)	10		6	1.40	(0.50	, 3.91)			
Stable cases															
Unstable cases															
95% CI															
Genotype	N		N	OR	Lower	Upper	Controls	N		OR	Lower	Upper			
GG	1059		77	1.00	-Ref-		111		27	3.36	(2.06	, 5.46)			0.18
GA	626		56	1.23	(0.85	, 1.76)	62		11	2.43	(1.22	, 4.84)			
AA	95		13	1.96	(1.04	, 3.69)	10		9	12.45	(4.85	, 32.0)			

Adjusted for sex, race, center and usual smoking amount. Number of subjects varies slightly from Table 1 due to missing data for one or more adjustment variables. Family history was defined as colorectal cancer reported in first-degree relatives.