Association of Smoking, CpG Island Methylator Phenotype, and V600E BRAF Mutations in Colon Cancer

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Background: **Cigarette smoking has been associated with microsatellite instability in sporadic colon cancer. Most microsatellite-unstable colon cancers have widespread methylation of CpG islands (i.e., the CpG island methylator phenotype [CIMP]), and many of these tumors harbor the V600E BRAF mutation. We investigated whether the association between smoking and all colon cancers could be explained through induction of CIMP and/or BRAF mutations.** ***Methods:*** **We evaluated 1315 case patients with colon cancer and 2392 control subjects in a population-based study. Demographic information, including smoking history, was obtained in an interview. Microsatellite instability was determined primarily by evaluation of the mononucleotide repeat BAT-26. CIMP was determined by sodium bisulfite modification of DNA followed by methylation-specific polymerase chain reaction amplification of CpG islands in hMLH1, p16, and MINTS1, -2, and -31. Tumors were scored as CIMP high (i.e., ≥2 CpG islands methylated) or CIMP low (i.e., <2 CpG islands methylated). BRAF V600E mutations were identified by sequencing. Logistic regression was used to quantify relationships among smoking, CIMP, and BRAF. All statistical tests were two-sided.** ***Results:*** **Heavy smoking (i.e., >20 cigarettes per day), compared with nonsmoking, was associated with an increased risk of CIMP-high colon cancer (odds ratio [OR] = 2.06, 95% confidence interval [CI] = 1.43 to 2.97) and also with BRAF V600E mutations (OR = 3.16, 95% CI = 1.80 to 5.54). The association between cigarette smoking and the risk of colon cancer was limited to the minority of tumors that were CIMP high and BRAF wild type or CIMP high and BRAF mutated (for heavy smokers, OR = 1.91, 95% CI = 1.23 to 2.97, and OR = 2.85, 95% CI = 1.53 to 5.29, respectively). All relationships above showed a statistically significant relationship to amount smoked (P_{trend}<.001 for all, except that relationship with tumors that were CIMP high and BRAF wild type, for which P_{trend} = .008) and were independent of microsatellite instability.** ***Conclusions:*** **Previously identified associations between smoking and colon cancer, whether microsatellite unstable or stable, appear to be explained by the association of smoking with CIMP and BRAF mutations. [J Natl Cancer Inst 2006;98:1731–8]**

Cigarette smoking has been associated with microsatellite instability in sporadic colon cancer *(1,2)*. Most colon cancers with microsatellite instability have widespread methylation of CpG islands, i.e., the so-called CpG island methylator phenotype (CIMP), and many harbor V600E BRAF mutations, as do a minority of microsatellite-stable tumors *(3)*. Smoking has been associated with CpG island methylation in bronchial epithelium and lung cancer *(4–9)*, and so the association of smoking with instability might be explained through the induction of CIMP and/or BRAF mutations. If this possibility were true, then relationships among cigarette smoking and CIMP and/or BRAF mutations among microsatellite-stable tumors should exist. In this study, we evaluate the relationship among cigarette smoking and CIMP and BRAF among colon cancer overall, among microsatellite-stable colon

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cancers and among microsatellite-unstable colon cancers. To our knowledge, risk factors for these epigenetic and genetic changes have not been previously reported.

Participants and Methods

Study Population and Other Data

We evaluated 1315 case patients with colon cancer and 2392 control subjects in a population-based study. Study participants were black, white, or Hispanic and were from the Kaiser Permanente Medical Care Program (KPMCP) of Northern California, an eight-county area in Utah (i.e., Davis, Salt Lake, Utah, Weber, Wasatch, Tooele, Morgan, and Summit Counties), or the Twin Cities Metropolitan area in Minnesota. Eligibility criteria for case patients included diagnosis with a first primary, incident colon cancer (International Classification of Diseases for Oncology, 2nd revision, codes 18.0 and 18.2–18.9) between October 1, 1991, and September 30, 1994; age between 30 and 79 years at time of diagnosis; and being mentally competent to complete the interview. Case eligibility was determined by the Surveillance, Epidemiology, and End Results Cancer Registries in Northern California and in Utah and through the Minnesota Surveillance System through rapid-reporting systems. The median time from diagnosis to interview was 131 days overall (126 days at KPMCP, 154 days in Minnesota, and 109 days in Utah). Case patients 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 disease were not eligible. Among all patients contacted, 75.6% participated. Those who did not participate were more likely to be older and to have higher stage tumors than those who participated *(10)*.

Control subjects were frequency matched to case patients by sex and by 5-year age groups. They were selected from eligibility lists for KPMCP; driver's license lists for Minnesota; and random digit dialing, driver's license lists, or Health Care Finance Administration lists for Utah, by the same eligibility criteria that were used for case patients. These methods have been described in detail *(11)*. Of all control subjects contacted, 63.7% participated.

Written informed consent was obtained from each participant. The studies were approved by the Utah, KPMCP, and Minnesota institutional review boards.

Environmental exposure data were collected by trained and certified interviewers *(11)*. The referent period for the study was the calendar year approximately 2 years before the date of diagnosis or of selection for the study. Information was collected on demographic factors (such as age, sex, and race), physical activity, body size (including height, usual adult weight, and weight 2 and 5 years before diagnosis), use of aspirin and/or nonsteroidal anti-inflammatory drugs, cigarette smoking history, and medical history. A measure of long-term (past 20 years) levels of vigorous leisure-time physical activity was used because this variable was shown to be a sensitive predictor of cancer risk in this population *(11)*. Body mass index, expressed as weight in kilograms/(height in meters)^2, was used as an indicator of body size. Participants were asked if they had ever smoked at least 100 cigarettes. Among those who reported smoking, the usual number of cigarettes smoked in a day was determined as part of the smoking history questionnaire. Previous studies using this dataset have shown that the number of cigarettes smoked per day is the most reliable measure of smoking in this population *(12)*. We also determined pack-years of smoking by asking the age an individual began smoking regularly, the age they stopped, and the number of cigarettes smoked per day. Both current and former smokers were included.

Detection of BRAF V600E Mutations

The method for detecting BRAF V600E mutations in this population has been described *(3)*. Briefly, exon 15 of BRAF was amplified by use of a polymerase chain reaction (PCR) from DNA previously extracted from tumor tissue that was microdissected from formalin-fixed, paraffin-embedded tissue blocks *(13)*, with the forward primer 5′-TCATAATGCTTGCTCT GATAGGA-3′ and the reverse primer 5′-CTTTCTAGTAACT CAGCAGC-3′. Amplifications were carried out with AmpliTaq Gold (Applied Biosystems, Foster City, CA) and a PCR profile consisting of a 9-minute initial denaturation at 95 °C; followed by 35 cycles of 20 seconds at 95 °C, 20 seconds at 60 °C, and 30 seconds at 72 °C; and with a 5-minute final extension at 72 °C. Mutations were verified by sequencing in both directions. Sufficient DNA was available for analysis from tumors of 1522 individuals, of whom 1271 had interview data and were therefore included in the analysis for association with smoking. BRAF mutations other than the V600E mutation were identified in four tumors, but we considered only the V600E mutation in statistical analyses because of uncertainty regarding the pathogenicity of these less common mutations.

CpG Island Methylator Phenotype Assays

CIMP assays have been described *(3)*. Briefly, DNA extracted from tumor tissue that was microdissected from formalin-fixed, paraffin-embedded blocks was modified with sodium bisulfite, followed by methylation-specific PCR *(14)*. Sodium bisulfite modification changes unmethylated cytosines to uracils; methylated cytosines, however, are protected from this modification. Methylation-specific PCR takes advantage of this observation by using a primer specific for a particular cytosine. The subsequent PCR then amplifies only DNA in which that cytosine is methylated and, therefore, unchanged by sodium bisulfite. This technique was used to amplify methylated CpG cites in the promoters of the genes hMLH1 and p16 as well as methylated CpG sites in DNA clones preferentially methylated in colon cancer, the so-called MINT (i.e., methylated in tumors) 1, 2, and 31 *(15,16)*. Tumors were scored as CIMP high if two or more of the CpG islands were methylated and as CIMP low if less than two were methylated *(15,17)*. Sufficient DNA was available for analysis of tumors from 1391 individuals, of whom 1143 had interview data and were therefore included in the analysis.

Clinicopathologic relationships of CIMP and BRAF mutations of these tumors have been described previously in detail *(3,18)*. This study included data for 940 participants studied in these previous reports (i.e., those who had interview data) and data for an additional 375 participants not included in the earlier reports, 312 of whom were from the Minnesota center, which was not included in the previous reports. Tumor DNA from the other 63 participants had been depleted by previous studies; DNA for these participants was obtained by acquiring new sections from tumor blocks, microdissecting the tumor, and

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extracting the DNA, as described previously *(19)*. In previous studies that did not require interview data, only samples from patients in Utah and Northern California were used *(3,18)* because the Minnesota institutional review board required the reconsent of patients to obtain tumor blocks, whereas the Utah and KPMCP institutional review boards did not. Thus, the samples from patients in Utah and KPMCP were more population-based than those of patients in Minnesota. Studies that required interview data, such as the analyses involving smoking, had fewer samples because not all individuals participated in the interview. We therefore included the samples from patients in Minnesota in this study to increase our sample size and our ability to detect associations. Adjustment for study center, however, did not affect the results presented in this study (center-adjusted data not shown).

Microsatellite Instability

Microsatellite instability, i.e., the expansion or contraction of short nucleotide repeats, was evaluated by PCR amplification of the respective repeat by use of flanking oligonucleotide primers. Instability at 12 such repeats (BAT-26, TGFβRII, and a panel of 10 tetranucleotide repeats) had been determined in a previous study on smoking and instability *(1)*. This previous study on microsatellite instability and smoking included 1510 subjects. The current study included 1290 of these 1510 subjects who had results for BRAF and/or CIMP in tumor tissue. For 1217 of the 1290 tumors, results from the mononucleotide repeat BAT-26, a good marker for generalized instability *(20)*, were used to score a tumor as stable or unstable. Sixty-nine tumors for which data for BAT-26 could not be obtained were classified by use of a mononucleotide repeat in the gene TGFβRII, another good indicator of generalized instability *(20)*. Four tumors for which data for both BAT-26 and TGFβRII could not be obtained were classified by use of the panel of 10 tetranucleotide repeats *(21)*. With this panel, a tumor was classified as unstable if instability was detected in 30% or more of the repeats and as stable if instability was present in less than 30%. The primer sequences and PCR conditions for amplification of all these repeats have been previously described *(21–23)*. We have previously shown *(20,24)* that BAT-26, TGFβRII, and the panel of 10 tetranucleotide repeats are strongly associated with the Bethesda consensus panel for microsatellite instability. Microsatellite instability could not be determined for 25 tumors because PCR amplification failed. We were unable to determine microstatellite instability status for 21 of the 1143 colon cancers with CIMP results, including five of the CIMP-high tumors. We were also unable to determine microsatellite instability status for 22 of the 1271 colon cancers with BRAF results, including three of the BRAF V600E–mutated tumors.

Statistical Methods

Smoking was defined as the usual number of cigarettes smoked per day (0, 1–20, or >20 cigarettes per day) or as pack-years (0, <30, or ≥30 pack-years; cut points were based upon the median values for control subjects). Multivariable logistic and polytomous (i.e., more than two outcomes for the dependent variable, also called polychotomous) logistic regression analyses were used to calculate odds ratios (ORs) for the association between cigarette smoking and CIMP-high or CIMP-low colon cancers, compared with control subjects, and adjusted for age at selection, body mass index, lifetime physical activity, long-term alcohol consumption, energy intake, dietary fiber consumption, dietary calcium level (all as continuous variables), aspirin or nonsteroidal anti-inflammatory drug use (three times a week usage for at least 1 month during the previous 2 years), and sex. Odds ratios for the association between cigarette smoking and colon cancers with or without a V600E BRAF mutation were calculated by the same approach. To compare subgroups of tumors directly, case–case logistic regression models were used to calculate odds ratios for CIMP status (high versus low) and for BRAF status (V600E mutant versus wild type). *P* values for trend in association were calculated by use of an exposure variable representing ordered categories of amount of smoking as a continuous variable. Univariate analysis of the number of CpG islands methylated and BRAF V600E mutation status among tumors was performed by logistic regression analysis. All data analyses were performed with SAS version 9.1 (SAS Institute, Cary, NC). All tests of statistical significance were two-sided. Results with men and women were generally similar and were therefore combined.

Results

Characteristics of control subjects and case patients with colon cancer, overall and with respect to CIMP and BRAF status, are shown in Table 1. CIMP-high status was present in 326 (28.5%) of 1143 colon cancers, including 207 (21.4%) of 969 microsatellite-stable cancers and 114 (74.5%) of 153 microsatellite-unstable cancers. BRAF V600E mutations were found in 123 (9.7%) of 1271 colon cancers, including 53 (4.9%) of 1079 microsatellite-stable cancers and 67 (39.4%) of 170 microsatellite-unstable cancers. Among microsatellite-stable tumors, more BRAF mutations were found in tumors with CIMP high (41 [89.1%] of 46 tumors) than in those with CIMP low (only 5 [0.68%] of 735 tumors). A similar pattern was observed among microsatellite-unstable tumors; more BRAF mutations were found in tumors with high CIMP (62 [95.4%] of 65 tumors) than in those with low CIMP (only 3 [7.9%] of 38 tumors). Thus, irrespective of instability status, BRAF mutations were mostly observed in CIMP-high tumors.

CpG Island Methylator Phenotype and Smoking

To investigate the association between smoking and CIMP status, we used case–control comparisons (case patients with colon cancer compared with control subjects without colon cancer) and case–case comparisons (case patients with colon cancer with or without CIMP compared). In case–control comparisons, cigarette smoking was associated with CIMP high for overall colon cancer, microsatellite-stable colon cancer, and microsatellite-unstable colon cancer; statistically significant dose–response relationships with respect to amount smoked were found for all groups (P_{trend}<.001, .007, and .005, respectively; Table 2). Smoking more than 20 cigarettes per day was associated with an approximately twofold increased risk of CIMP-high cancer, compared with nonsmoking (OR = 2.06, 95% confidence interval [CI] = 1.43 to 2.97); however, no association was found between smoking and CIMP-low colon cancers, overall or with or without microsatellite stability. A similar analysis that used the number of pack-years smoked (0, <30, or ≥30 pack-years) instead of the number of cigarettes per day showed nearly identical

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Table 1. Description of case patients and control subjects*

Characteristic	Control subjects, No. (%)	Case patients				
		All, No. (%)	Low CIMP, No. (%)	High CIMP, No. (%)	Wt BRAF, No. (%)	Mut BRAF, No. (%)
Total	2392	1315	817 (71.5)	326 (28.5)	1148 (90.3)	123 (9.7)
Age, y						
<55	416 (17.4)	212 (16.1)	149 (18.2)	33 (10.1)	200 (17.4)	9 (7.3)
55–64	567 (23.7)	349 (26.5)	226 (27.7)	79 (24.2)	302 (26.3)	30 (24.4)
65–70	593 (24.8)	304 (23.1)	195 (23.9)	70 (21.5)	262 (22.8)	32 (26.0)
71–79	816 (34.1)	450 (34.2)	247 (30.2)	144 (44.2)	384 (33.4)	52 (42.3)
Center						
KPMCP	1021 (42.7)	685 (52.1)	425 (52.0)	158 (48.5)	614 (53.5)	52 (42.3)
Minnesota	858 (35.9)	357 (27.1)	221 (27.1)	91 (27.9)	295 (25.7)	46 (37.4)
Utah	513 (21.4)	273 (20.8)	171 (20.9)	77 (23.6)	239 (20.8)	25 (20.3)
Sex						
Female	1109 (46.4)	598 (45.5)	348 (42.6)	164 (50.3)	514 (44.8)	62 (50.4)
Male	1283 (53.6)	717 (54.5)	469 (57.4)	162 (49.7)	634 (55.2)	61 (49.6)
Race						
White, non-Hispanic	2199 (92.0)	1165 (88.7)	716 (87.6)	298 (91.7)	1007 (87.8)	116 (94.3)
White Hispanic	106 (4.4)	63 (4.8)	37 (4.5)	16 (4.9)	59 (5.1)	4 (3.3)
Black, non-Hispanic	82 (3.4)	81 (6.2)	62 (7.6)	9 (2.8)	78 (6.8)	1 (0.8)
Black Hispanic	3 (0.1)	3 (0.2)	1 (0.1)	2 (0.6)	2 (0.2)	1 (0.8)
Other	1 (0.0)	2 (0.2)	1 (0.1)	0 (0.0)	1 (0.1)	1 (0.8)
Site of tumor						
Distal		618 (47.0)	465 (56.9)	76 (23.3)	587 (51.1)	17 (13.8)
Proximal		668 (50.8)	335 (41.0)	241 (73.9)	536 (46.7)	102 (82.9)
Unknown		29 (2.2)	17 (2.1)	9 (2.8)	25 (2.2)	4 (3.3)
AJCC stage						
1		327 (25.1)	225 (27.8)	67 (20.6)	291 (25.6)	22 (18.0)
2		414 (31.8)	239 (29.6)	105 (32.3)	356 (31.3)	43 (35.2)
3		405 (31.1)	242 (30.0)	119 (36.6)	351 (30.8)	44 (36.1)
4		157 (12.0)	102 (12.6)	34 (10.5)	140 (12.3)	13 (10.7)

*CIMP = CpG island methylator phenotype; Wt = wild type; Mut = mutant; KPMCP = Kaiser Permanente Medical Care Program; AJCC = American Joint Committee on Cancer.

results (data not shown). In a case–case comparison of tumors with or without CIMP high, cigarette smoking was also found to have statistically significant dose–response relationships with CIMP-high colon cancers overall and among microsatellite-stable cancers (P_{trend} = .003 and .04, respectively). Smoking more than 20 cigarettes per day was associated with a nearly twofold higher risk of a CIMP-high cancer than that of a CIMP-low cancer (OR = 1.79, 95% CI = 1.19 to 2.70). In the case–case comparison among patients with microsatellite-unstable cancers, the association between cigarette smoking and CIMP-high status was stronger than that among patients with microsatellite-stable cancers (for those smoking >20 cigarettes per day, OR = 2.32, 95% CI = 0.60 to 8.93, versus OR = 1.63, 95% CI = 1.01 to 2.63), but the P_{trend} values were not statistically significant in this smaller group of

Table 2. Association of cigarette smoking with CIMP*

Instability status by cigarettes smoked, No. per day	Control subjects, No. (%)	Case patients		CIMP-high vs. control subjects, OR (95% CI)	CIMP-low vs. control subjects, OR (95% CI)	CIMP high vs. CIMP low subjects, OR (95% CI)
		CIMP high, No. (%)	CIMP low, No. (%)			
Overall colon cancers						
None	1113 (46.5)	125 (38.3)	368 (45.0)	1.00 (referent)	1.00 (referent)	1.00 (referent)
≤20 cigarettes	956 (40.0)	135 (41.4)	308 (37.7)	1.36 (1.03 to 1.81)	0.94 (0.78 to 1.14)	1.44 (1.06 to 1.98)
>20 cigarettes	323 (13.5)	66 (20.2)	141 (17.3)	2.06 (1.43 to 2.97)	1.17 (0.90 to 1.51)	1.79 (1.19 to 2.70)
P_{trend}†				<.001	.42	.003
Microsatellite-stable colon cancers						
None	1113 (46.5)	82 (39.6)	344 (45.1)	1.00 (referent)	1.00 (referent)	1.00 (referent)
≤20 cigarettes	956 (40.0)	83 (40.1)	285 (37.4)	1.28 (0.91 to 1.80)	0.93 (0.77 to 1.13)	1.35 (0.93 to 1.97)
>20 cigarettes	323 (13.5)	42 (20.3)	133 (17.5)	1.88 (1.21 to 2.92)	1.18 (0.91 to 1.53)	1.63 (1.01 to 2.63)
P_{trend}†				.007	.41	.04
Microsatellite-unstable colon cancers						
None	1113 (46.5)	41 (36.0)	19 (48.7)	1.00 (referent)	1.00 (referent)	1.00 (referent)
≤20 cigarettes	956 (40.0)	50 (43.9)	15 (38.5)	1.54 (0.97 to 2.45)	0.78 (0.37 to 1.62)	1.96 (0.76 to 5.06)
>20 cigarettes	323 (13.5)	23 (20.2)	5 (12.8)	2.36 (1.30 to 4.29)	0.67 (0.23 to 1.99)	2.32 (0.60 to 8.93)
P_{trend}†				.005	.41	.14

*OR = odds ratio; CI = confidence interval; CIMP = CpG island methylator phenotype.

†Trend test and P_{trend} were from a logistic regression model that was adjusted for age, sex, body mass index, physical activity, alcohol, aspirin, and/or nonsteroidal anti-inflammatory drug use, calories, dietary fiber, and calcium. All statistical tests were two-sided.

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Table 3. Association of cigarette smoking with BRAF V600E mutations*

Instability status by cigarettes smoked, No. per day	Control subjects, No. (%)	Case patients		BRAF Mut vs. control subjects, OR (95% CI)	BRAF Wt vs. control subjects, OR (95% CI)	BRAF Mut vs. BRAF Wt, OR (95% CI)
		BRAF Mut, No. (%)	BRAF Wt, No. (%)			
Overall colon cancers						
None	1113 (46.5)	38 (30.9)	499 (43.5)	1.00 (referent)	1.00 (referent)	1.00 (referent)
≤20 cigarettes	956 (40.0)	57 (46.3)	446 (38.9)	1.99 (1.26 to 3.13)	1.04 (0.88 to 1.22)	1.92 (1.21 to 3.04)
>20 cigarettes	323 (13.5)	28 (22.8)	203 (17.7)	3.16 (1.80 to 5.54)	1.32 (1.05 to 1.66)	2.46 (1.37 to 4.42)
P_{trend}†				<.001	.04	.001
Microsatellite-stable colon cancers						
None	1113 (46.5)	15 (28.3)	450 (43.9)	1.00 (referent)	1.00 (referent)	1.00 (referent)
≤20 cigarettes	956 (40.0)	26 (49.1)	391 (38.1)	2.35 (1.18 to 4.66)	1.02 (0.85 to 1.21)	2.23 (1.12 to 4.43)
>20 cigarettes	323 (13.5)	12 (22.6)	185 (18.0)	3.37 (1.44 to 7.85)	1.35 (1.06 to 1.70)	2.50 (1.06 to 5.91)
P_{trend}†				.003	.04	.02
Microsatellite-unstable colon cancers						
None	1113 (46.5)	22 (32.8)	42 (40.8)	1.00 (referent)	1.00 (referent)	1.00 (referent)
≤20 cigarettes	956 (40.0)	30 (44.8)	47 (45.6)	1.81 (0.99 to 3.31)	1.11 (0.69 to 1.76)	1.53 (0.68 to 3.46)
>20 cigarettes	323 (13.5)	15 (22.4)	14 (13.6)	3.00 (1.42 to 6.37)	0.97 (0.49 to 1.90)	2.81 (0.91 to 8.70)
P_{trend}†				.004	.96	.07

*OR = odds ratio; CI = confidence interval; Mut = mutant; Wt = wild type.

†Trend test and P_{trend} were from a logistic regression model that was adjusted for age, sex, body mass index, physical activity, alcohol, aspirin, and/or nonsteroidal anti-inflammatory drug use, calories, dietary fiber, and calcium. All statistical tests were two-sided.

case patients. In conclusion, cigarette smoking was associated with CIMP-high status in both case–control and case–case comparisons.

BRAF Status and Smoking

To investigate the association between BRAF status and smoking, we used case–control comparisons (case patients with colon cancer compared with control subjects without colon cancer) and case–case comparisons (case patients with colon cancer with or without BRAF mutations compared). In case–control comparisons, cigarette smoking was associated with the BRAF V600E mutation status among patients with colon cancers irrespective of instability status, and statistically significant dose–response relationships with respect to amount smoked were observed for colon cancer overall, microsatellite-stable cancers, and microsatellite-unstable cancers (P_{trend}<.001, P = .003, and P = .004, respectively; Table 3). Smoking more than 20 cigarettes per day was associated with an approximately threefold higher risk of colon cancer with a BRAF V600E mutation as compared with nonsmoking (OR = 3.16, 95% CI = 1.80 to 5.54). Smoking was also associated with an increased risk of colon cancers overall and microsatellite-stable colon cancers without BRAF mutations, with statistically significant dose–response relationships (P = .04), although the point estimates were less than those found for BRAF-mutated cancers (for those smoking >20 cigarettes per day, OR = 1.32, 95% CI = 1.05 to 1.66, for colon cancer overall). In case–case comparisons, cigarette smoking was more strongly associated with BRAF-mutated colon cancers than with BRAF wild-type colon cancers overall and among microsatellite-stable cancers, with statistically significant dose–response relationships (P_{trend} = .001 and .02, respectively). Smoking more than 20 cigarettes per day was associated with a more than twofold risk of colon cancer with a BRAF V600E mutation (OR = 2.46, 95% CI = 1.37 to 4.42) compared with colon cancers with wild-type BRAF. Similar but nonstatistically significant associations were found among microsatellite-unstable cancers in the comparison of BRAF-mutated versus wild-type BRAF cancers. In conclusion, cigarette smoking was associated with BRAF V600E mutations in both case–control and case–case comparisons.

Combined BRAF and CpG Island Methylator Phenotype Analysis

As noted above, most BRAF mutations occurred in tumors that were CIMP high. A polytomous analysis of the relationship between cigarette smoking and colon cancers with various combinations of CIMP and BRAF mutations, compared with control subjects without colon cancer, was performed to more precisely delineate the effects of smoking (Table 4). The largest group consisted of patients with CIMP-low colon cancers that had wild-type BRAF; among these patients, cigarette smoking was not associated with an increased risk of colon cancer. Smoking was associated with an increased risk of CIMP-high colon cancers with wild-type BRAF, both overall (for those smoking >20 cigarettes per day, OR = 1.91, 95% CI = 1.23 to 2.97) and for microsatellite-stable cancer (OR = 2.02, 95% CI = 1.25 to 3.27); statistically significant dose–response relationships with respect to amount smoked were observed (P_{trend} = .008 and .009, respectively). Cigarette smoking was also associated with CIMP-high colon cancer with mutant BRAF, overall (for those smoking >20 cigarettes per day, OR = 2.85, 95% CI = 1.53 to 5.29) and for microsatellite-unstable cancer (OR = 3.43, 95% CI = 1.57 to 7.50); statistically significant dose–response relationships were also observed (P_{trend}<.001 and P = .002, respectively). Finally, smoking was associated with an approximately twofold increased risk of CIMP-high colon cancer with mutant BRAF and microsatellite stability (for those smoking >20 cigarettes per day, OR = 1.92, 95% CI = 0.67 to 5.51), but the trend for the dose–response relationship was not statistically significant (P = .12). In conclusion, cigarette smoking was associated with an increased risk of colon cancers that are CIMP high and BRAF wild type and that are CIMP high and BRAF mutant, and the association between

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Table 4. Association of cigarette smoking with combined BRAF and CIMP status*

Instability status by cigarettes smoked, No. per day	Control subjects, No. (%)	CIMP low + BRAF Wt, No. (%)	CIMP high + BRAF Wt, No. (%)	CIMP high + BRAF Mut, No. (%)	CIMP-low + BRAF Wt vs. control subjects, OR (95% CI)	CIMP-high + BRAF Wt vs. control subjects, OR (95% CI)	CIMP-high + BRAF Mut vs. control subjects, OR (95% CI)
Overall colon cancers							
None	1113 (46.5)	351 (45.1)	82 (39.6)	35 (33.0)	1.00 (referent)	1.00 (referent)	1.00 (referent)
≤20 cigarettes	956 (40.0)	295 (37.9)	82 (39.6)	50 (47.2)	0.96 (0.79 to 1.17)	1.23 (0.87 to 1.73)	2.01 (1.24 to 3.23)
>20 cigarettes	323 (13.5)	132 (17.0)	43 (20.8)	21 (19.8)	1.18 (0.90 to 1.53)	1.91 (1.23 to 2.97)	2.85 (1.53 to 5.29)
P_{trend}†					.38	.008	<.001
Microsatellite-stable colon cancers							
None	1113 (46.5)	329 (45.1)	64 (39.5)	14 (34.1)	1.00 (referent)	1.00 (referent)	1.00 (referent)
≤20 cigarettes	956 (40.0)	275 (37.7)	62 (38.3)	21 (51.2)	0.96 (0.79 to 1.17)	1.20 (0.81 to 1.77)	2.10 (1.00 to 4.38)
>20 cigarettes	323 (13.5)	126 (17.3)	36 (22.2)	6 (14.6)	1.20 (0.92 to 1.57)	2.02 (1.25 to 3.27)	1.92 (0.67 to 5.51)
P_{trend}†					.32	.009	.12
Microsatellite-unstable colon cancers							
None	1113 (46.5)	17 (48.6)	17 (39.5)	20 (32.3)	1.00 (referent)	1.00 (referent)	1.00 (referent)
≤20 cigarettes	956 (40.0)	14 (40.0)	19 (44.2)	28 (45.2)	0.83 (0.39 to 1.78)	1.19 (0.57 to 2.47)	1.96 (1.04 to 3.68)
>20 cigarettes	323 (13.5)	4 (11.4)	7 (16.3)	14 (22.6)	0.63 (0.19 to 2.08)	1.41 (0.52 to 3.82)	3.43 (1.57 to 7.50)
P_{trend}†					.40	.50	.002

*OR = odds ratio; CI = confidence interval; CIMP = CpG island methylator phenotype; WT = wild type; Mut = mutant.

†Trend test and P_{trend} were from a logistic regression model that was adjusted for age, sex, body mass index, physical activity, alcohol, aspirin, and/or nonsteroidal anti-inflammatory drug use, calories, dietary fiber, and calcium. All statistical tests were two-sided.

smoking and colon cancer appears to be largely explained by its association with CIMP and/or BRAF status.

Methylation and BRAF

It is possible that stronger relationships observed between smoking and BRAF than those observed between smoking and CIMP (Tables 2 and 3) could reflect higher degrees of CpG island methylation in BRAF-mutated tumors. Indeed, the number of methylated CpG islands in BRAF-mutated tumors was significantly higher than that in BRAF wild-type tumors, regardless of the instability status of the tumor (Table 5; P_{trend}<.001).

Discussion

In this study, we observed that cigarette smoking was associated with CIMP-high colon cancer irrespective of microsatellite instability status, with statistically significant dose–response relationships with respect to amount smoked. Cigarette smoking was also associated with BRAF mutations in colon cancer. Cigarette smoking has been associated with microsatellite instability in colon cancer *(1,2)*, and microsatellite-unstable cancers are frequently CIMP high and harbor the V600E BRAF mutation *(3)*. The relationships that we found among microsatellite-stable cancers between cigarette smoking and CIMP status or BRAF mutations indicate that smoking may be associated with most microsatellite-unstable tumors and a small subset of microsatellite-stable cancers through a mechanism involving methylation and/or BRAF mutations. This possibility was also supported by the analysis of the effect of smoking on the risk of various combinations of CIMP and BRAF alterations in patients with colon cancer compared with control subjects. No statistically significant association was found between smoking and CIMP-low tumors with wild-type BRAF, the largest subset of colon cancers. Only cancers (either microsatellite stable or unstable) with CIMP-high or both CIMP-high and BRAF mutations were associated with smoking, indicating that the increased risk of colon cancer associated with smoking may be largely explained by the association between smoking and CIMP and BRAF status. Although a previous study *(25)* reported nonstatistically significant trends among dietary folate and alcohol intake and promoter methylation of certain genes, risk factors for CpG island methylation as a global phenotype and/or BRAF mutations, to our knowledge, have not been previously reported.

Previous studies *(12,26–28)*, which did not take into account these acquired genetic and epigenetic changes, found either no association between smoking and colon cancer or only a weak association, with risk estimates of 1.3–1.4. These risk estimates

Table 5. Association between the degree of methylation and BRAF mutations in CIMP-high tumors*

No. of CIMP markers methylated	Overall colon cancers			Microsatellite stable colon cancers			Microsatellite unstable colon cancers		
	BRAF Wt, No. (%)	BRAF Mut, No. (%)	OR (95% CI)	BRAF Wt, No. (%)	BRAF Mut, No. (%)	OR (95% CI)	BRAF Wt, No. (%)	BRAF Mut, No. (%)	OR (95% CI)
2	102 (57.0)	8 (8.5)	1.00 (referent)	92 (64.8)	8 (21.6)	1.00 (referent)	9 (25.7)	0 (0.0)	1.00 (referent)
3	42 (23.5)	13 (13.8)	3.91 (1.39 to 11.74)	32 (22.5)	13 (35.1)	4.61 (1.60 to 14.13)	9 (25.7)	0 (0.0)	Undefined
4	24 (13.4)	33 (35.1)	17.12 (6.73 to 48.66)	15 (10.6)	14 (37.8)	10.44 (3.43 to 34.30)	9 (25.7)	17 (30.9)	20.35 (2.78 to ∞)
5	11 (6.1)	40 (42.6)	43.25 (15.49 to 137.27)	3 (2.1)	2 (5.4)	7.38 (0.54 to75.14)	8 (22.9)	38 (69.1)	49.52 (6.93 to ∞)
P_{trend}†			<.001			<.001			<.001

*OR = odds ratio; CI = confidence interval; CIMP = CpG island methylator phenotype; WT = wild type; Mut = mutant.

†Trend test and P_{trend} were from an unadjusted exact logistic regression model. All statistical tests were two-sided.

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are, in fact, similar to those that we observed for colon cancers without BRAF mutations (for those smoking >20 cigarettes per day, OR = 1.35, 95% CI = 1.06 to 1.70), compared with control subjects without colon cancer. This increased risk, however, may be the result of including CIMP-high tumors in the wild-type BRAF group because most CIMP-high tumors (as we have defined CIMP) have a BRAF wild-type status *(3)*. By defining colon cancers with respect to CIMP and BRAF status, we could observe stronger associations that were specific to the subset of tumors with these genetic and epigenetic alterations.

Although the precise mechanism between smoking and CpG island methylation is currently unclear, this association has been found in studies *(4–9,29)* of bronchial epithelium or non–small-cell lung cancers in humans and in experimental animals. Compounds in cigarette smoke activate the aromatic hydrocarbon receptor, and recent studies *(30,31)* have shown that activation of this receptor leads to methylation of the p16 and p53 promoters in human keratinocytes. The relationship between smoking and BRAF mutations may also involve CpG island methylation. We found that 93% of all BRAF-mutated tumors in this study had a CIMP-high status and that, among such tumors, those with mutated BRAF typically had higher levels of methylation than those with wild-type BRAF. It is therefore possible that the stronger associations observed between smoking and BRAF mutations than those between smoking and CIMP reflect the higher degree of CpG island methylation observed in BRAF-mutated tumors and that the general mechanism behind our observations is induction of CpG island methylation by smoking.

Other techniques and/or panels of CpG islands that can be used to determine CIMP status *(32)* may strengthen relationships to smoking. However, the associations that we found between smoking and CIMP support the hypothesis that CIMP is a true colon cancer phenotype, which is still somewhat controversial *(3,33,34)*.

It could be argued that, among patients with microsatellite-unstable cancers, the relationship between smoking and BRAF and/or CIMP status may, in part, be explained by including some patients with hereditary nonpolyposis colon cancer (HNPCC) in the non-CIMP, wild-type BRAF, microsatellite-unstable group. Because very few, if any, HNPCC-associated tumors have a CIMP-high status or BRAF mutations *(35–37)* and because CIMP-high and BRAF mutations are relatively frequent epigenetic and genetic events in sporadic microsatellite-unstable tumors, it would be difficult to separate the association of smoking with CIMP and/or BRAF status from the association of smoking with microsatellite instability. The association of smoking with BRAF and CIMP status among patients with microsatellite-stable tumors more specifically supports a general carcinogenic mechanism in which smoking induces a CIMP-high status and/or BRAF mutations in colon cancers. Thus, this mechanism also appears to be a likely explanation for the association between smoking and microsatellite instability.

A potential source of bias in this study involves relationships among tumor stage, smoking, and CIMP and/or BRAF status. We have previously observed *(3,18)* that, in microsatellite-stable tumors, both CIMP and BRAF mutations were more likely to be found in higher stage tumors than in lower stage tumors. If smoking was also related to advanced tumor stage, then the association between smoking and these acquired changes (i.e., CIMP and BRAF mutations) in tumors could result from their common association with tumor stage. However, in a previous study *(10)* in this population, we observed that smoking was actually associated with lower stage tumors, and adjustment for tumor stage in the current study did not change our results (data not shown).

There are several limitations to this study. First, as with most epidemiologic studies, our results demonstrate an association but should not be viewed as causal. It is encouraging, however, that there is evidence both in animals and humans linking smoking with CpG methylation *(4–9,29)*. Second, data were collected retrospectively, and participants were asked to recall their cigarette smoking history. It is possible that this recollection could have introduced a reporting error; however, associations observed with cigarette smoking for colon cancer overall were similar to those reported in other prospective and retrospective studies *(38)*. Finally, our population was mainly non-Hispanic white, and it is unknown whether associations would be similar for other ethnic groups. Thus, our results require validation by other studies and in other ethnic groups.

The associations that we observed between an exposure—cigarette smoking—and specific molecular markers—CIMP and BRAF status—in tumor tissue provide further evidence that colon cancer develops through more than one etiologic and/or molecular pathway. These results also illustrate how stratification of tumors on molecular characteristics can provide clues to the mechanisms within each pathway and reveal associations with risk factors heretofore obscured by the genetic heterogeneity of cancer.

In summary, cigarette smoking appeared to be associated with an increased risk of colon cancer with CIMP and/or BRAF V600E mutations, irrespective of microsatellite instability status, indicating that the original observation of an association of smoking with microsatellite instability *(1)* may be attributed to the association of smoking with CIMP and BRAF status. Our results also suggest that relatively weak, previously identified associations between smoking and colon cancer may be attributed to the relatively strong association of smoking with the small subset of colon cancers that have a CIMP-high or BRAF-mutated status.

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Notes

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