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Original Contribution

A Prospective Study of Duration of Smoking Cessation and Colorectal Cancer Risk by Epigenetics-related Tumor Classification

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The effect of duration of cigarette smoking cessation on colorectal cancer risk by molecular subtypes remains unclear. Using duplication-method Cox proportional-hazards regression analyses, we examined associations between duration of smoking cessation and colorectal cancer risk according to status of CpG island methylator phenotype (CIMP), microsatellite instability, v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) mutation, or DNA methyltransferase-3B (DNMT3B) expression. Follow-up of 134,204 individuals in 2 US nationwide prospective cohorts (Nurses' Health Study (1980–2008) and Health Professionals Follow-up Study (1986–2008)) resulted in 1,260 incident rectal and colon cancers with available molecular data. Compared with current smoking, 10–19, 20–39, and ≥40 years of smoking cessation were associated with a lower risk of CIMP-high colorectal cancer, with multivariate hazard ratios (95% confidence intervals) of 0.53 (0.29, 0.95), 0.52 (0.32, 0.85), and 0.50 (0.27, 0.94), respectively ($P_{trend} = 0.001$), but not with the risk of CIMP-low/CIMP-negative cancer ($P_{trend} = 0.25$) ($P_{heterogeneity} = 0.02$, between CIMP-high and CIMP-low/CIMP-negative cancer risks). Differential associations between smoking cessation and cancer risks by microsatellite instability ($P_{heterogeneity} = 0.02$), DNMT3B expression ($P_{heterogeneity} = 0.03$), and *BRAF* ($P_{heterogeneity} = 0.10$) status appeared to be driven by the associations of CIMP-high cancer with microsatellite instability–high, DNMT3B-positive, and *BRAF*-mutated cancers. These molecular pathological epidemiology data suggest a protective effect of smoking cessation on a DNA methylation–related carcinogenesis pathway leading to CIMP-high colorectal cancer.

carcinogen; carcinoma; hypermethylation; epigenomics; molecular epidemiology; public health; tobacco; translational epidemiology

Abbreviations: CI, confidence interval; CIMP, CpG island methylator phenotype; DNMT3B, DNA methyltransferase 3B; HR, hazard ratio; MPE, molecular pathological epidemiology; MSI, microsatellite instability.

Smoking is a risk factor for several cancers, including colorectal cancer, and remains a global health problem (1, 2). Although the carcinogenic effect of smoking is not refutable, the effect of duration of smoking cessation on colorectal cancer risk remains unclear. Beyond a simple comparison of former versus current smokers, some epidemiologic studies suggest a modest association between duration of smoking cessation and risk reduction in overall colorectal cancer incidence compared with continued smoking (3, 4), whereas other studies did not confirm this association (5–7).

Colorectal cancers are a heterogeneous group of neoplasms displaying a complex mixture of epigenetic and genetic alterations (8). Molecular classification of colorectal cancer has become crucial for epidemiologic research and clinical decision making (8–11). The CpG island methylator phenotype (CIMP) is a form of epigenomic instability characterized by widespread promoter CpG island hypermethylation

(12–16), and microsatellite instability (MSI) represents a distinct form of genomic instability (8, 17). A high degree of CIMP in colorectal cancer (CIMP-high) is associated with v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*) oncogene mutation as well as a high degree of MSI (through epigenetic silencing of *MLH1*) (13–15, 18–20). Experimental and observational evidence suggests that DNA methyltransferase-3B (DNMT3B) expression could contribute to CIMP in colorectal cancer (21–25). Epidemiologic studies suggest that cigarette smoking is associated with higher risks for specific molecular subtypes of colorectal cancer—namely, CIMP-high (26–28), MSI-high (26, 28–34), and *BRAF*-mutated (26–28, 35) cancers. However, to our knowledge, no previous study has prospectively examined duration of smoking cessation and colorectal cancer incidence by tumor epigenetic subtyping. Experimental evidence suggests that cigarette smoking could affect epigenetic status and induce hypermethylation in CpG islands (36–38). Therefore, we hypothesized that duration of smoking cessation might be associated specifically with a decreased risk of CIMP-high colorectal cancer.

We conducted a molecular pathological epidemiology (MPE) (10, 11) study to prospectively examine the relation between duration of smoking cessation and colorectal cancer risk by epigenetics-related tumor classifications, including status of CIMP, MSI, *BRAF* mutation, and DNMT3B expression. Studies have shown that these tumor molecular features are interrelated (13–15, 18–28, 34, 35). For this purpose, we used tumor specimens of 1,260 incident colorectal cancer cases from 2 US nationwide prospective cohort studies with more than 134,000 participants.

MATERIALS AND METHODS

Study population

Details on our study population are described in the Web Appendix (available at http://aje.oxfordjournals.org/). Briefly, we used the Nurses' Health Study and the Health Professionals Follow-up Study (39, 40). Questionnaires were sent to participants every 2 years to update information on smoking status and other lifestyle factors. A total of 88,397 women and 45,807 men were eligible for inclusion in the analysis. Informed consent was obtained from all participants. This study was approved by the Human Subjects Committees at Harvard School of Public Health and Brigham and Women's Hospital.

Assessment of smoking status

Details on the method used to obtain information on smoking have been reported previously (41, 42). Current smoking status and the number of cigarettes smoked per day were reported by participants on questionnaires updated every 2 years, beginning in 1980 for women and in 1986 for men. In addition, at the cohort baseline questionnaires, we collected information on age when smoking was started, age when smoking was stopped (for former smokers), and pack-years smoked before age 30 years. Thus, we could calculate the duration of smoking cessation and cumulative pack-years smoked (cumulative average of packs per day × the number of years during which smoking occurred).

Assessment of incident colorectal cancer

Details on the assessment of incident colorectal cancer are described in the Web Appendix. Briefly, we obtained the information from biennial questionnaires, medical records, and the National Death Index (43). On the basis of the colorectal continuum model, we used both colon and rectal cancers as outcomes (43, 44). We retrieved formalin-fixed paraffin-embedded colorectal cancer tissue blocks from hospitals throughout the United States at which participants with colorectal cancer had undergone surgical resection (45).

Assessment of tumor characteristics

Detailed methods of the assessment of tumor characteristics are described in the Web Appendix. We conducted DNA extraction, Pyrosequencing of *BRAF* (codon 600) (46), MSI analysis (20), and methylation analysis for 8 CpG islands (18, 20, 47), using validated bisulfite DNA treatment and real-time polymerase chain reaction (MethyLight assay) (48). We performed immunohistochemistry for DNMT3B (22).

Statistical methods

We used Cox proportional-hazards model to estimate hazard ratios, with adjustment for multiple potential confounders. For each 2-year interval, we used the most up-to-date questionnaire data for all covariates before the next follow-up cycle. We treated all variables as time-dependent variables to take into account changes over time (39). Follow-up ended at diagnosis of colorectal cancer, death from other causes, or June 30, 2008, whichever came first. To reduce within-individual variation and to better estimate long-term influence, we used cumulative average for relevant variables, which was the mean of all available data up to before each biennial follow-up cycle (39). Covariates included body mass index (weight (kg)/height $(m)^2$; <25 vs. 25–30 vs. ≥30); history of colorectal cancer in any first-degree relative (yes vs. no); regular use of aspirin (2 or more tablets per week or at least 2 times per week vs. less); physical activity level (quintiles of mean metabolic equivalent task hours per week); alcohol consumption (0 gram per day or quartiles of grams per day); total caloric intake (quintiles of calories per day) and red meat intake (quintiles of servings per day). Models were stratified with calendar year of the questionnaire cycle, age in month, and sex (only in combined cohorts). We observed no evidence for a violation of the proportional hazard assumption on the basis of the interaction terms between smoking status and follow-up time ($P > 0.1$ for all the combination of smoking variables and colorectal cancer outcomes). The linear trend test was conducted by using the median value of each category. We examined the possibly nonlinear relation between years of smoking cessation and colorectal cancer risk by molecular subtypes nonparametrically using restricted cubic splines (49). To compare differential associations of smoking with colorectal cancer risk by molecular subtypes, we conducted duplication-method Cox proportional hazards model (50). This methodology permits the estimation of separate regression coefficients for smoking status stratified by the type of outcome. Using a likelihood ratio test, we examined

whether smoking conferred differential risk by molecular subtype (e.g., CIMP-low/negative vs. CIMP-high). All *P* values were two-sided. All statistical analyses were performed using SAS version 9.2 (SAS Institute, Inc., Cary, North Carolina). No attempt was made to adjust for multiple testing because of difficulty in determining the number of independent hypotheses tested (i.e., the smoking indicators were related and the tumor biomarkers were related). Nonetheless, statistical significance was evaluated cautiously considering the exploratory nature of the analyses and the number of biomarkers analyzed.

RESULTS

Table 1 shows the age-adjusted baseline characteristics of the study population in the Nurses' Health Study and the Health Professionals Follow-up Study. The rate of restart of smoking was 1.5%–1.2% in 1980s and decreased in recent years (0.7%–0.5% in 2000s). We identified 1,260 incident colorectal cancers with available pathological specimens suitable for molecular analysis, during follow-up of 134,204 individuals (3,101,031 person-years). There were 205 (18% of 1,170) CIMP-high tumors, 188 (16% of 1,200) MSI-high tumors, 178 (15% of 1,218) *BRAF*-mutated tumors, and 108 (15% of 728; DNMT3B data were limited to those included in tissue microarray) DNMT3B-positive tumors. The relations between tumor molecular features, tumor location, and sex are shown in Web Table 1.

Web Table 2 shows cohort (sex)-specific results for smoking cessation and incident colorectal cancer risk by molecular subtypes. We conducted tests of heterogeneity using the Q statistic and observed no significant heterogeneity between the 2 cohorts ($P_{\text{heterogeneity}} \geq 0.05$) for the associations of smoking cessation with any of the specific cancer subtypes. For further analyses, we utilized the combined cohorts to increase statistical power.

In the combined cohorts, compared with current smoker, duration of smoking cessation was not significantly associated with the risk of colorectal cancer overall (Table 2). Although smoking cessation appeared to be more protective for proximal colon cancer than for distal colorectal cancer, the difference was not statistically significant ($P_{\text{heterogeneity}} = 0.28$) (Table 2). Web Table 3 shows the risk for proximal colon cancer and distal colorectal cancer by molecular subtypes; the statistical power was limited in these subsite-specific analyses.

Duration of smoking cessation and colorectal cancer risk by molecular subtypes

Compared with current smokers, duration of smoking cessation was associated with a significantly reduced risk of CIMP-high colorectal cancer ($P_{\text{trend}} = 0.001$). Compared with current smokers, multivariate hazard ratios for smoking cessation of 10–19, 20–39, and ≥40 years were 0.53 (95% confidence interval (CI): 0.29, 0.95), 0.52 (95% CI: 0.32, 0.85), and 0.50 (95% CI: 0.27, 0.94), respectively (Table 2). Approximately 50% lower risk of CIMP-high cancer among former smokers with long-term cessation (compared with current smokers) was similar to the risk of CIMP-high cancer among never smokers compared with current smokers (hazard ratio (HR) = 0.47; 95% CI: 0.31, 0.73; for never smokers compared with current smokers; HR = 2.08; 95% CI: 1.35, 3.20; for current smokers compared with never smokers). In contrast, smoking cessation was not significantly associated with CIMP-low/negative cancer risk ($P_{\text{trend}} = 0.25$), and the association of smoking cessation with the cancer risk significantly differed by CIMP status ($P_{\text{heterogeneity}} = 0.02$).

Longer duration of smoking cessation was associated with a decrease in MSI-high cancer risk ($P_{\text{trend}} = 0.002$), but was not significantly associated with microsatellite-stable cancer risk ($P_{\text{trend}} = 0.36$; $P_{\text{heterogeneity}} = 0.02$) (Table 2). Longer duration of smoking cessation was associated with a decreased risk for DNMT3B-positive cancer ($P_{\text{trend}} = 0.01$), but not with DNTM3B-negative cancer risk ($P_{\text{trend}} = 0.61$; $P_{\text{heterogeneity}} = 0.03$) (Table 2). The association of smoking cessation with cancer risk did not significantly differ by *BRAF* mutation status ($P_{\text{heterogeneity}} = 0.10$).

Smoothing spline plots (Web Figure 1) show dose-response relation between the duration of smoking cessation and a decrease in the risk of CIMP-high, MSI-high, or DNMT3B-positive cancers. Web Table 4 shows the risk estimates for duration of smoking cessation compared with never smokers.

Smoking cessation and risk of combined molecular subtypes

Because CIMP-high is associated with MSI-high and DNMT3B-positive status in colorectal cancer (13–15, 18–20), we examined combined molecular features, to assess which molecular subtype risk was reduced by smoking cessation independent of other molecular features. This combined analysis was conducted using the molecular features which were significantly associated with smoking cessation in Table 2, and could confound each other. Compared with current smokers, the risk reduction associated with smoking cessation was apparent for CIMP-high cancers regardless of MSI status ($P_{\text{trend}} \leq 0.02$), and CIMP-high cancers regardless of DNMT3B status ($P_{\text{trend}} \leq 0.02$) (Table 3). In analysis using combined *BRAF* and CIMP status, the relation between smoking cessation and CIMP-high cancer risk was apparent irrespective of *BRAF* mutation status (data not shown). The findings suggest that risk reduction associated with smoking cessation might be present primarily on CIMP-high cancer.

Smoking cessation and tumor molecular subtypes in strata of cumulative pack-years smoked

We examined the association of smoking cessation with the risk for specific cancer subtypes in strata of cumulative pack-years smoked, in an attempt to control for confounding by cumulative pack-years. Among current/former smokers with 20 or more pack-years, longer duration of cessation was associated with significantly lower risk for CIMP-high cancer ($P_{\text{trend}} = 0.02$), and DNMT3B-positive cancer ($P_{\text{trend}} = 0.04$) (Web Table 5). The association of smoking cessation with colorectal cancer risk differed significantly by CIMP status ($P_{\text{heterogeneity}} = 0.02$) and DNMT3B expression status

Table 1. Age-adjusted Characteristics of Participants During Follow-up[a] According to Smoking Status in the Nurses' Health Study (1980–2008) and the Health Professionals Follow-up Study (1986–2008)

Variable	Women (Nurses' Health Study)								Men (Health Professionals Follow-up Study)							
	Never Smoker (*n* = 38,576)		Former Smoker				Current Smoker (*n* = 25,592)		Never Smoker (*n* = 21,366)		Former Smoker				Current Smoker (*n* = 4,627)	
			Cessation for <10 Years (*n* = 14,289)		Cessation for ≥10 Years (*n* = 9,940)						Cessation for <10 Years (*n* = 13,880)		Cessation for ≥10 Years (*n* = 5,934)			
	%	Mean (SD)	%	Mean (SD)	%	Mean (SD)	%	Mean (SD)	%	Mean (SD)	%	Mean (SD)	%	Mean (SD)	%	Mean (SD)
Total person-years		511,458		325,952		124,626		191,635		188,401		156,652		36,951		30,770
Age[b]		68.9 (8.7)		70.0 (7.9)		68.7 (8.3)		68.1 (8.0)		61.1 (11.0)		65.1 (10.4)		59.8 (10.4)		59.8 (9.8)
Body mass index[c]																
<25	70		70		74		79		69		66		64		71	
25–29.9	21		21		19		16		27		29		30		25	
≥30	9		9		7		5		5		5		6		4	
Family history of colorectal cancer in any first-degree relative	13		13		12		11		12		12		11		11	
Regular use of aspirin	40		42		43		42		45		49		49		45	
Postmenopausal hormone use (ever)	63		68		63		56		N/A		N/A		N/A		N/A	
Physical activity, MET-hours/week[d]		15.8 (17.5)		17.3 (19.4)		15.6 (18.6)		13.5 (17.6)		31.3 (29.4)		30.2 (28.1)		25.2 (25.2)		23.0 (24.5)
Alcohol consumption, g/day		3.8 (7.0)		7.1 (9.3)		7.8 (10.4)		9.1 (12.4)		7.9 (11.1)		13.1 (14.6)		14.7 (16.3)		16.8 (18.8)
Total calories, kcal/day		1,697 (449)		1,672 (430)		1,638 (439)		1,637 (463)		1,985 (554)		1,966 (549)		1,970 (571)		2,012 (589)
Red meat intake, servings/day		1.1 (0.6)		1.0 (0.6)		1.1 (0.6)		1.2 (0.7)		1.1 (0.8)		1.1 (0.8)		1.3 (0.9)		1.5 (0.9)
Cumulative pack-years		N/A		13.1 (13.5)		29.6 (20.8)		40.3 (21.8)		N/A		19.5 (15.6)		32.0 (22.5)		39.7 (24.6)
Pack-years smoked before age 30[b]		N/A		6.8 (6.1)		6.3 (5.0)		6.7 (4.4)		N/A		10.6 (6.9)		9.9 (6.7)		10.2 (6.6)
<20 years of age[b] at start of smoking, %		N/A	59		56		58			N/A	54		50		51	

Abbreviations: MET, metabolic equivalent task; N/A, not applicable; SD, standard deviation.

[a] Updated information of smoking status from biennial questionnaires was averaged, using person-years in each category of smoking status up to censoring (including death from other causes) or immediately before personal colorectal cancer diagnosis if it occurred. Values were standardized to the age distribution of the study population.

[b] Not age-adjusted.

[c] Weight (kg)/height $(m)^2$.

[d] MET calculated according to the frequency of a range of physical activities in 1986 for both women and men.

Table 2. Duration of Smoking Cessation and Incident Colorectal Cancer Risk by Molecular Subtypes[a] in the Nurses' Health Study (1980–2008) and the Health Professionals Follow-up Study (1986–2008)

	Current Smoker (n = 439,508 person-years)		Cessation for 1–4 Years (n = 161,905 person-years)		Cessation for 5–9 Years (n = 155,720 person-years)		Cessation for 10–19 Years (n = 312,757 person-years)		Cessation for 20–39 Years (n = 511,426 person-years)		Cessation for ≥40 Years (n = 126,688 person-years)		P_{trend}[b]	$P_{heterogeneity}$[c]
	HR	95% CI	HR	95% CI	HR	95% CI	HR	95% CI	HR	95% CI	HR	95% CI		
Cancers														
All colorectal cancer														
No.	139		60		86		129		242		105			
Age-adjusted	1.00	Referent	0.98	0.72, 1.33	1.31	1.00, 1.71	0.96	0.75, 1.22	0.92	0.74, 1.13	1.02	0.78, 1.33	0.19	
Multivariate[d]	1.00	Referent	0.99	0.73, 1.34	1.30	0.99, 1.71	0.96	0.75, 1.23	0.92	0.74, 1.14	1.05	0.80, 1.37	0.29	
Proximal colon cancer														
No.	63		32		42		57		109		51			
Age-adjusted	1.00	Referent	1.16	0.76, 1.78	1.32	0.89, 1.96	0.86	0.60, 1.24	0.81	0.59, 1.12	0.82	0.55, 1.21	0.01	
Multivariate[d]	1.00	Referent	1.17	0.76, 1.80	1.32	0.89, 1.96	0.86	0.60, 1.24	0.81	0.59, 1.12	0.84	0.57, 1.24	0.02	0.28
Distal colorectal cancer														
No.	75		28		43		72		129		52			
Age-adjusted	1.00	Referent	0.82	0.53, 1.27	1.26	0.86, 1.84	1.03	0.74, 1.43	0.90	0.67, 1.20	0.95	0.65, 1.38	0.28	
Multivariate[d]	1.00	Referent	0.83	0.54, 1.29	1.26	0.86, 1.84	1.03	0.74, 1.43	0.90	0.67, 1.21	0.96	0.66, 1.41	0.34	
CIMP Status														
CIMP-low/negative														
No.	103		42		66		105		194		72			
Age-adjusted	1.00	Referent	0.91	0.63, 1.31	1.37	1.00, 1.87	1.07	0.81, 1.41	0.97	0.76, 1.24	0.93	0.68, 1.28	0.17	
Multivariate[d]	1.00	Referent	0.92	0.64, 1.32	1.37	1.00, 1.88	1.07	0.81, 1.42	0.98	0.77, 1.26	0.95	0.69, 1.32	0.25	0.02
CIMP-high														
No.	31		15		15		18		37		16			
Age-adjusted	1.00	Referent	1.09	0.58, 2.02	0.89	0.48, 1.66	0.52	0.29, 0.93	0.52	0.32, 0.84	0.48	0.26, 0.90	0.001	
Multivariate[d]	1.00	Referent	1.12	0.60, 2.08	0.89	0.48, 1.67	0.53	0.29, 0.95	0.52	0.32, 0.85	0.50	0.27, 0.94	0.001	
MSI Status														
MSS														
No.	108		40		68		101		201		86			
Age-adjusted	1.00	Referent	0.83	0.57, 1.19	1.34	0.98, 1.82	0.97	0.74, 1.28	0.93	0.73, 1.19	0.96	0.71, 1.30	0.26	
Multivariate[d]	1.00	Referent	0.83	0.58, 1.20	1.34	0.98, 1.82	0.97	0.73, 1.28	0.94	0.74, 1.20	0.98	0.72, 1.33	0.36	0.02
MSI-high														
No.	27		16		14		20		30		17			
Age-adjusted	1.00	Referent	1.27	0.68, 2.37	0.97	0.51, 1.86	0.66	0.37, 1.19	0.50	0.29, 0.84	0.60	0.31, 1.13	0.001	
Multivariate[d]	1.00	Referent	1.29	0.69, 2.40	0.96	0.50, 1.84	0.67	0.37, 1.20	0.50	0.29, 0.85	0.62	0.33, 1.17	0.002	

Table continues

Table 2. Continued

	Current Smoker (n = 439,508 person-years)		Cessation for 1–4 Years (n = 161,905 person-years)		Cessation for 5–9 Years (n = 155,720 person-years)		Cessation for 10–19 Years (n = 312,757 person-years)		Cessation for 20–39 Years (n = 511,426 person-years)		Cessation for ≥40 Years (n = 126,688 person-years)		P_{trend}[b]	$P_{heterogeneity}$[c]
	HR	95% CI	HR	95% CI	HR	95% CI	HR	95% CI	HR	95% CI	HR	95% CI		
							BRAF *Mutation Status*							
BRAF-wildtype														
No.	114		42		70		105		207		89			
Age-adjusted	1.00	Referent	0.81	0.57, 1.16	1.28	0.95, 1.73	0.93	0.71, 1.21	0.88	0.69, 1.11	0.89	0.66, 1.19	0.12	
Multivariate[d]	1.00	Referent	0.82	0.57, 1.17	1.28	0.95, 1.73	0.93	0.71, 1.21	0.88	0.70, 1.12	0.91	0.67, 1.22	0.18	0.10
BRAF-mutated														
No.	22		14		13		19		30		13			
Age-adjusted	1.00	Referent	1.47	0.75, 2.89	1.19	0.60, 2.37	0.87	0.47, 1.63	0.73	0.42, 1.28	0.76	0.37, 1.56	0.02	
Multivariate[d]	1.00	Referent	1.48	0.75, 2.91	1.17	0.59, 2.34	0.88	0.47, 1.64	0.73	0.41, 1.28	0.77	0.38, 1.59	0.02	
							DNMT3B Expression Status							
DNMT3B-negative														
No.	73		35		38		72		123		37			
Age-adjusted	1.00	Referent	1.10	0.73, 1.65	1.15	0.77, 1.70	1.17	0.84, 1.63	1.02	0.76, 1.37	0.96	0.63, 1.47	0.40	
Multivariate[d]	1.00	Referent	1.11	0.74, 1.66	1.15	0.77, 1.71	1.19	0.85, 1.65	1.04	0.77, 1.41	1.01	0.66, 1.54	0.61	0.03
DNMT3B-positive														
No.	17		5		8		5		16		5			
Age-adjusted	1.00	Referent	0.76	0.28, 2.07	0.99	0.42, 2.32	0.32	0.12, 0.87	0.50	0.25, 1.01	0.43	0.15, 1.23	0.01	
Multivariate[d]	1.00	Referent	0.78	0.28, 2.12	1.00	0.43, 2.34	0.33	0.12, 0.90	0.52	0.26, 1.05	0.44	0.15, 1.25	0.01	

Abbreviations: CI, confidence interval; CIMP, CpG island methylator phenotype; DNMT3B, DNA methyltransferase 3B; HR, hazard ratio; MSI, microsatellite instability; MSS, microsatellite stable.

[a] All models were stratified by calendar year of the questionnaire cycle, age, and sex.

[b] Based on the linear trend test across the median values in each category. To test whether the duration of smoking cessation reduced the cancer risk compared with current smoking, trend tests and heterogeneity tests were performed on current and past smokers, excluding never smokers.

[c] Tests for heterogeneity (for a multivariate HR linear trend) showed significance of differential association of cessation with colorectal cancer risk by molecular subtypes (i.e., CIMP-low/negative vs. CIMP-high; MSS vs. MSI-high; *BRAF*-wildtype vs. *BRAF*-mutated; DNMT3B-negative vs. DNMT3B-positive).

[d] Models were adjusted for body mass index, family history of colorectal cancer in any first-degree relative, regular use of aspirin, physical activity level, alcohol consumption, total caloric intake, and red meat intake.

Table 3. Duration of Smoking Cessation and Colorectal Cancer Risk by Combined Molecular Subtypes[a] in the Nurses' Health Study (1980–2008) and the Health Professionals Follow-up Study (1986–2008)

	Current Smoker		Cessation for 1–4 Years		Cessation for 5–9 Years		Cessation for ≥10 Years		P_{trend}[b]
	HR	95% CI	HR	95% CI	HR	95% CI	HR	95% CI	
CIMP/MSI Subtyping									
CIMP-low/negative									
MSS									
No.	94		37		60		346		
Age-adjusted	1.00	Referent	0.88	0.60, 1.29	1.37	0.99, 1.90	1.02	0.80, 1.29	0.66
Multivariate[c]	1.00	Referent	0.88	0.60, 1.29	1.37	0.99, 1.90	1.03	0.81, 1.30	0.81
MSI-high									
No.	6		3		2		13		
Age-adjusted	1.00	Referent	1.13	0.28, 4.59	0.71	0.14, 3.56	0.57	0.21, 1.56	0.08
Multivariate[c]	1.00	Referent	1.15	0.28, 4.68	0.70	0.14, 3.52	0.58	0.21, 1.58	0.08
CIMP-high									
MSS									
No.	11		2		3		19		
Age-adjusted	1.00	Referent	0.45	0.10, 2.05	0.52	0.14, 1.88	0.37	0.17, 0.80	0.02
Multivariate[c]	1.00	Referent	0.47	0.10, 2.14	0.54	0.15, 1.94	0.38	0.18, 0.83	0.02
MSI-high									
No.	20		13		12		50		
Age-adjusted	1.00	Referent	1.40	0.69, 2.82	1.08	0.52, 2.22	0.56	0.33, 0.96	0.002
Multivariate[c]	1.00	Referent	1.43	0.71, 2.88	1.07	0.52, 2.20	0.57	0.33, 0.97	0.003
CIMP/DNMT3B Subtyping									
CIMP-low/negative									
DNMT3B-negative									
No.	56		27		32		206		
Age-adjusted	1.00	Referent	1.10	0.69, 1.75	1.27	0.82, 1.97	1.25	0.92, 1.69	0.28
Multivariate[c]	1.00	Referent	1.10	0.69, 1.75	1.28	0.82, 1.98	1.27	0.93, 1.73	0.20
DNMT3B-positive									
No.	10		2		6		19		
Age-adjusted	1.00	Referent	0.52	0.11, 2.41	1.33	0.48, 3.70	0.55	0.25, 1.22	0.06
Multivariate[c]	1.00	Referent	0.53	0.11, 2.44	1.32	0.47, 3.69	0.56	0.25, 1.25	0.07
CIMP-high									
DNMT3B-negative									
No.	14		6		3		21		
Age-adjusted	1.00	Referent	0.95	0.36, 2.49	0.40	0.12, 1.41	0.41	0.20, 0.82	0.02
Multivariate[c]	1.00	Referent	0.98	0.37, 2.57	0.41	0.12, 1.42	0.42	0.21, 0.85	0.02
DNMT3B-positive									
No.	7		3		2		7		
Age-adjusted	1.00	Referent	1.07	0.27, 4.17	0.55	0.11, 2.67	0.28	0.10, 0.81	0.01
Multivariate[c]	1.00	Referent	1.12	0.29, 4.38	0.56	0.12, 2.73	0.29	0.10, 0.85	0.01

Abbreviations: CI, confidence interval; CIMP, CpG island methylator phenotype; DNMT3B, DNA methyltransferase 3B; HR, hazard ratio; MSI, microsatellite instability; MSS, microsatellite stable.

[a] All models were stratified by calendar year of the questionnaire cycle, age, and sex.

[b] Based on the linear trend test by using the median value of each category. To test whether the duration of smoking cessation reduced the cancer risk compared with current smoking, trend test and heterogeneity tests were performed on current and past smokers, excluding never smokers.

[c] Models were adjusted for body mass index, family history of colorectal cancer in any first-degree relative, regular use of aspirin, physical activity level, alcohol consumption, total caloric intake, and red meat intake.

($P_{heterogeneity} = 0.03$). Statistical power was limited in the stratum of <20 pack-years.

Other smoking variables and colorectal cancer risk by molecular subtypes

We examined the association between other smoking indicators (including cumulative pack-years, pack-years smoked before age 30, and age at start of smoking) and colorectal cancer risk by molecular subtypes separately women and men (Web Tables 6 and 7), and among the combined cohorts (Tables 4 and 5). The category of never smokers was used as the referent group because we attempted to see whether smoking increased the risk of specific cancer subtype. Compared with never smokers, smoking of 40 or more pack-years was associated with higher risks of CIMP-high cancer (multivariate HR = 2.12; 95% CI: 1.48, 3.03; $P_{trend} < 0.0001$), MSI-high cancer (multivariate HR = 2.27; 95% CI: 1.56, 3.31; $P_{trend} < 0.0001$), and *BRAF*-mutated cancer (multivariate HR = 2.00; 95% CI: 1.37, 2.92; $P_{trend} = 0.0001$) (Table 4). In contrast, cumulative pack-years were not significantly associated with the risk of CIMP-low/negative cancer, microsatellite-stable cancer, or *BRAF*-wildtype cancer ($P_{trend} \geq 0.10$). The association of cumulative pack-years with the cancer risk differed by CIMP status ($P_{heterogeneity} = 0.001$), MSI status ($P_{heterogeneity} = 0.0003$), and *BRAF* mutation status ($P_{heterogeneity} = 0.01$). The relation between cumulative pack-years and cancer risk did not significantly differ by DNMT3B status ($P_{heterogeneity} = 0.83$).

Because CIMP-high is associated with both MSI-high and *BRAF* mutation in colorectal cancer (13–15, 18–20), we examined the relation between cumulative pack-years and cancer risk by combined molecular subtyping (Table 6). Combined molecular analysis was conducted using the molecular features which were significantly associated with cumulative pack-years in Table 4, and could confound each other. In CIMP/MSI subtyping, compared with never smokers, 40 or more pack-years smoked were associated with a higher risk for CIMP-high/MSI-high cancer (multivariate HR = 2.75; 95% CI: 1.78, 4.26; $P_{trend} < 0.0001$), but not with the other 3 CIMP/MSI subtypes ($P_{trend} \geq 0.15$).

In CIMP/*BRAF* subtyping, cumulative pack-years was significantly associated with a higher risk for CIMP-high cancer regardless of *BRAF* status ($P_{trend} \leq 0.03$), but not with CIMP-low/negative cancer risk ($P_{trend} \geq 0.15$). In MSI/*BRAF* subtyping, cumulative pack-years smoked was significantly associated with a higher risk for MSI-high cancer regardless of *BRAF* status ($P_{trend} \leq 0.03$), but not with microsatellite-stable cancer risk ($P_{trend} \geq 0.24$).

DISCUSSION

We conducted this unique analysis to prospectively examine the relation between duration of smoking cessation and colorectal cancer risk by molecularly-defined subtypes. We utilized 2 US nationwide prospective cohort studies with available lifestyle information, including smoking status at multiple time points during follow-up, as well as tumor molecular data. We showed that, compared with current smokers, duration of smoking cessation was associated with a decreased risk of CIMP-high colorectal cancer (but not with the risk of CIMP-low/negative cancer). There might be a plateau of the effect of cessation duration beyond 10 years, as risk estimates were similar beyond 10 years of cessation (multivariate HRs of 0.50–0.53, compared with current smoking). Our data suggest that smoking cessation might be effective in preventing specific molecular subtypes of colorectal cancer. Our data also underscore the importance of cessation in as early as possible, because, after 10 years of cessation, the CIMP-high cancer risk appeared to be almost similar to never smokers.

We observed a significant trend of risk reduction for proximal colon cancer but not for distal colorectal cancer; this anatomical difference in cancer risk might be due to higher prevalence of CIMP-high in proximal colon cancers (43, 44). Considering the "colorectal continuum" hypothesis (43, 44), the effect of smoking and its cessation might continuously change along the bowel subsites. Additional studies are necessary to examine the effect of smoking on carcinogenesis in detailed colorectal subsites.

Molecular features of colorectal cancer such as CIMP-high, MSI-high, *BRAF* mutations and DNMT3B expression are known to be interrelated (13–15, 18–25). Smoking cessation was associated with lower risks of MSI-high and DNMT3B-positive colorectal cancers, and these associations appeared to be driven by CIMP-high cancers enriched in these molecular subtypes. The well-documented association between smoking and *BRAF*-mutated cancer (26–28, 35) might be due to enrichment of the CIMP-high subtype in the *BRAF*-mutated cancers. Therefore, our current analysis emphasizes the importance of considering influence of multiple molecular features on epidemiologic associations (so-called "molecular confounding" (51)).

The relation between smoking and a specific cancer epigenotype is plausible. Cigarette smoke contains over 4,000 toxic chemicals, many of which can induce DNA damage (52). Evidence suggests that cigarette smoking and nicotine can induce DNA methylation (36–38, 53, 54). Changes in DNA methylation could be observed within 9 months after cigarette smoke condensate was applied to human epithelial cells (37). Additional studies are needed to elucidate the exact mechanisms of effects of smoking on epigenetic alterations.

Our present study represents MPE research (10, 11, 55). MPE is based on the unique tumor principle (51, 56) and etiologic heterogeneity according to molecular subtypes (e.g., CIMP-high vs. non-CIMP-high). Thus, MPE differs from conventional molecular epidemiology which typically deals with "colon cancer" as a single entity (57–59). MPE analysis can not only refine risk estimates for specific subtypes of cancer, but also provide evidence for causality and insights into pathogenic mechanisms (10, 11, 51, 60–65). We previously discussed how MPE research can provide evidence for causality in depth (10, 11). For example, although traditional epidemiology research has linked smoking to colorectal cancer, effect size for overall colorectal cancer risk by smoking has been modest (hazard ratio of about 1.2–1.3). In contrast, MPE research can find a consistent link between smoking and CIMP-high colorectal cancers with an accurate and substantial effect estimate for the CIMP-high subtype (hazard ratio of almost 2). This consistent link can provide further evidence for causality. The MPE approach enabled us to

Table 4. Smoking Status, Cumulative Pack-years of Smoking, and Incident Colorectal Cancer Risk by Molecular Subtypes[a] in the Nurses' Health Study (1980–2008) and the Health Professionals Follow-up Study (1986–2008)

	Smoking Status								Cumulative Pack-years of Smoking							
	Never (*n* = 1,383,154 person-years)		Former (*n* = 1,278,369 person-years)		Current (*n* = 439,508 person-years)		P_{trend}[b]	$P_{heterogeneity}$[c]	1–19 (*n* = 844,894 person-years)		20–39 (*n* = 511,272 person-years)		≥40 (*n* = 338,416 person-years)		P_{trend}[b]	$P_{heterogeneity}$[c]
	HR	95% CI	HR	95% CI	HR	95% CI			HR	95% CI	HR	95% CI	HR	95% CI		
All colorectal cancer																
No.	490		631		139				300		226		216			
Age-adjusted	1.00	Referent	1.23	1.09, 1.38	1.23	1.02, 1.49	0.001		1.09	0.94, 1.26	1.22	1.04, 1.43	1.35	1.15, 1.59	<0.0001	
Multivariate[d]	1.00	Referent	1.18	1.05, 1.34	1.17	0.96, 1.43	0.02		1.06	0.91, 1.23	1.17	0.99, 1.38	1.28	1.08, 1.51	0.002	
CIMP status								0.04								0.001
CIMP-low/negative																
No.	377		485		103				244		178		148			
Age-adjusted	1.00	Referent	1.21	1.06, 1.39	1.17	0.94, 1.47	0.02		1.15	0.98, 1.35	1.20	1.00, 1.44	1.20	0.99, 1.46	0.04	
Multivariate[d]	1.00	Referent	1.17	1.02, 1.35	1.12	0.89, 1.41	0.07		1.12	0.95, 1.32	1.16	0.97, 1.39	1.14	0.94, 1.39	0.15	
CIMP-high																
No.	71		103		31				34		36		56			
Age-adjusted	1.00	Referent	1.34	0.99, 1.81	2.19	1.43, 3.37	0.001		0.87	0.58, 1.31	1.37	0.91, 2.05	2.23	1.57, 3.18	<0.0001	
Multivariate[d]	1.00	Referent	1.30	0.95, 1.76	2.08	1.35, 3.20	0.002		0.86	0.57, 1.29	1.31	0.87, 1.96	2.12	1.48, 3.03	<0.0001	
MSI status								0.03								0.0003
MSS																
No.	400		504		108				254		175		159			
Age-adjusted	1.00	Referent	1.17	1.02, 1.34	1.19	0.96, 1.48	0.02		1.12	0.95, 1.31	1.10	0.92, 1.32	1.21	1.00, 1.45	0.06	
Multivariate[d]	1.00	Referent	1.13	0.99, 1.30	1.14	0.91, 1.42	0.09		1.09	0.93, 1.28	1.06	0.89, 1.28	1.15	0.95, 1.39	0.21	
MSI-high																
No.	63		98		27				34		37		50			
Age-adjusted	1.00	Referent	1.46	1.06, 2.01	2.16	1.36, 3.41	0.001		0.98	0.65, 1.49	1.60	1.06, 2.41	2.36	1.62, 3.44	<0.0001	
Multivariate[d]	1.00	Referent	1.42	1.03, 1.95	2.05	1.29, 3.26	0.002		0.96	0.63, 1.47	1.52	1.01, 2.30	2.27	1.56, 3.31	<0.0001	
BRAF mutation status								0.63								0.01
BRAF-wildtype																
No.	404		522		114				261		187		164			
Age-adjusted	1.00	Referent	1.20	1.05, 1.36	1.28	1.03, 1.58	0.003		1.14	0.97, 1.33	1.16	0.97, 1.38	1.24	1.03, 1.49	0.02	
Multivariate[d]	1.00	Referent	1.16	1.01, 1.32	1.22	0.98, 1.52	0.02		1.11	0.95, 1.30	1.11	0.93, 1.33	1.18	0.98, 1.43	0.10	
BRAF-mutated																
No.	67		89		22				31		28		48			
Age-adjusted	1.00	Referent	1.28	0.93, 1.76	1.43	0.87, 2.33	0.08		0.83	0.54, 1.27	1.19	0.76, 1.85	2.08	1.43, 3.03	<0.0001	
Multivariate[d]	1.00	Referent	1.24	0.90, 1.71	1.38	0.84, 2.25	0.13		0.81	0.53, 1.25	1.15	0.73, 1.79	2.00	1.37, 2.92	0.0001	

Table continues

Table 4. Continued

	Smoking Status								Cumulative Pack-years of Smoking							
	Never (n = 1,383,154 person-years)		Former (n = 1,278,369 person-years)		Current (n = 439,508 person-years)		P_{trend}[b]	$P_{heterogeneity}$[c]	1–19 (n = 844,894 person-years)		20–39 (n = 511,272 person-years)		≥40 (n = 338,416 person-years)		P_{trend}[b]	$P_{heterogeneity}$[c]
	HR	95% CI	HR	95% CI	HR	95% CI			HR	95% CI	HR	95% CI	HR	95% CI		
DNMT3B expression status								0.38								0.83
DNMT3B-negative																
No.	238		309		73				160		104		103			
Age-adjusted	1.00	Referent	1.26	1.06, 1.49	1.16	0.89, 1.51	0.05		1.21	0.99, 1.48	1.11	0.88, 1.40	1.29	1.02, 1.63	0.07	
Multivariate[d]	1.00	Referent	1.22	1.02, 1.45	1.10	0.84, 1.45	0.13		1.19	0.97, 1.46	1.08	0.85, 1.36	1.22	0.96, 1.55	0.19	
DNMT3B-positive																
No.	52		39		17				15		16		23			
Age-adjusted	1.00	Referent	0.69	0.46, 1.05	1.31	0.75, 2.28	0.95		0.51	0.28, 0.90	0.79	0.45, 1.39	1.26	0.77, 2.08	0.28	
Multivariate[d]	1.00	Referent	0.67	0.44, 1.02	1.22	0.69, 2.13	0.76		0.50	0.28, 0.89	0.75	0.42, 1.32	1.18	0.71, 1.95	0.42	

Abbreviations: CI, confidence interval; CIMP, CpG island methylator phenotype; DNMT3B, DNA methyltransferase 3B; HR, hazard ratio; MSI, microsatellite instability; MSS, microsatellite stable.

[a] All models were stratified by calendar year of the questionnaire cycle, age, and sex.

[b] Based on the linear trend test by using the median value of each category.

[c] Tests for heterogeneity (for a multivariate HR linear trend) of the associations of smoking with one molecular subtype versus the other molecular subtype (i.e., CIMP-low/negative vs. CIMP-high; MSS vs. MSI-high; *BRAF*-wildtype vs. *BRAF*-mutated; DNMT3B-negative vs. DNMT3B-positive).

[d] Models were adjusted for body mass index, family history of colorectal cancer in any first-degree relative, regular use of aspirin, physical activity level, alcohol consumption, total caloric intake, and red meat intake.

Table 5. Pack-years of Smoking Before Age 30 Years, Age at Start of Smoking, and Incident Colorectal Cancer Risk by Molecular Subtypes[a] in the Nurses' Health Study (1980–2008) and the Health Professionals Follow-up Study (1986–2008)

	Never Smoker (n = 1,383,154 person-years)		Pack-years Smoked Before Age 30 Years						Age at Start of Smoking, years					
			1–9 (n = 1,085,062 person-years)		≥10 (n = 560,470 person-years)		P_{trend}[b]	$P_{heterogeneity}$[c]	≥20 (n = 744,382 person-years)		<20 (n = 976,780 person-years)		P_{trend}[b]	$P_{heterogeneity}$[c]
	HR	95% CI	HR	95% CI	HR	95% CI			HR	95% CI	HR	95% CI		
All colorectal cancer														
No.	490		414		300				347		402			
Age-adjusted	1.00	Referent	1.09	0.96, 1.25	1.31	1.14, 1.52	0.0002		1.16	1.01, 1.33	1.21	1.06, 1.38	0.004	
Multivariate[d]	1.00	Referent	1.05	0.92, 1.20	1.25	1.08, 1.45	0.003		1.12	0.97, 1.29	1.16	1.01, 1.33	0.03	
CIMP status								0.09						0.17
CIMP-low/negative														
No.	377		322		222				274		301			
Age-adjusted	1.00	Referent	1.16	1.00, 1.35	1.13	0.95, 1.34	0.09		1.19	1.01, 1.39	1.16	0.99, 1.35	0.05	
Multivariate[d]	1.00	Referent	1.12	0.96, 1.31	1.07	0.90, 1.28	0.30		1.15	0.98, 1.35	1.11	0.95, 1.30	0.18	
CIMP-high														
No.	71		74		50				57		71			
Age-adjusted	1.00	Referent	1.33	0.96, 1.85	1.62	1.11, 2.35	0.01		1.27	0.89, 1.80	1.50	1.08, 2.09	0.02	
Multivariate[d]	1.00	Referent	1.29	0.93, 1.79	1.54	1.06, 2.25	0.02		1.23	0.87, 1.75	1.44	1.03, 2.01	0.03	
MSI status								0.05						0.21
MSS														
No.	400		323		240				275		318			
Age-adjusted	1.00	Referent	1.10	0.95, 1.28	1.13	0.96, 1.33	0.10		1.11	0.95, 1.29	1.15	0.99, 1.33	0.07	
Multivariate[d]	1.00	Referent	1.07	0.92, 1.24	1.07	0.91, 1.27	0.32		1.08	0.92, 1.26	1.10	0.94, 1.28	0.23	
MSI-high														
No.	63		75		45				63		60			
Age-adjusted	1.00	Referent	1.58	1.12, 2.21	1.61	1.09, 2.40	0.01		1.62	1.14, 2.30	1.45	1.01, 2.06	0.03	
Multivariate[d]	1.00	Referent	1.53	1.09, 2.15	1.55	1.04, 2.31	0.01		1.57	1.11, 2.24	1.39	0.97, 1.99	0.06	
BRAF mutation status								0.39						0.73
BRAF-wildtype														
No.	404		334		253				289		328			
Age-adjusted	1.00	Referent	1.14	0.99, 1.32	1.16	0.99, 1.37	0.03		1.15	0.99, 1.34	1.17	1.01, 1.36	0.03	
Multivariate[d]	1.00	Referent	1.11	0.95, 1.28	1.11	0.94, 1.31	0.15		1.12	0.96, 1.31	1.12	0.97, 1.31	0.12	
BRAF-mutated														
No.	67		68		37				53		56			
Age-adjusted	1.00	Referent	1.25	0.89, 1.75	1.35	0.89, 2.04	0.08		1.30	0.90, 1.86	1.24	0.87, 1.78	0.21	
Multivariate[d]	1.00	Referent	1.21	0.86, 1.71	1.29	0.85, 1.96	0.13		1.26	0.88, 1.82	1.20	0.83, 1.72	0.30	
DNMT3B expression status								0.41						0.07

Table continues

Table 5. Continued

	Never Smoker (n = 1,383,154 person-years)		Pack-years Smoked Before Age 30 Years						Age at Start of Smoking, years					
			1–9 (n = 1,085,062 person-years)		≥10 (n = 560,470 person-years)		P_{trend}[b]	$P_{heterogeneity}$[c]	≥20 (n = 744,382 person-years)		<20 (n = 976,780 person-years)		P_{trend}[b]	$P_{heterogeneity}$[c]
	HR	95% CI	HR	95% CI	HR	95% CI			HR	95% CI	HR	95% CI		
DNMT3B-negative														
No.	238		222		131				179		191			
Age-adjusted	1.00	Referent	1.23	1.02, 1.48	1.09	0.87, 1.35	0.25		1.21	1.00, 1.48	1.17	0.97, 1.42	0.08	
Multivariate[d]	1.00	Referent	1.20	0.99, 1.45	1.04	0.83, 1.30	0.54		1.18	0.97, 1.45	1.13	0.92, 1.37	0.21	
DNMT3B-positive														
No.	52		27		24				27		28			
Age-adjusted	1.00	Referent	0.67	0.42, 1.08	0.93	0.56, 1.53	0.72		0.82	0.52, 1.32	0.76	0.48, 1.21	0.23	
Multivariate[d]	1.00	Referent	0.65	0.41, 1.04	0.87	0.53, 1.45	0.53		0.80	0.50, 1.28	0.72	0.45, 1.15	0.15	

Abbreviations: CI, confidence interval; CIMP, CpG island methylator phenotype; DNMT3B, DNA methyltransferase 3B; HR, hazard ratio; MSI, microsatellite instability; MSS, microsatellite stable.

[a] All models were stratified by calendar year of the questionnaire cycle, age, and sex.

[b] Based on the linear trend test by using the median value of each category.

[c] Tests for heterogeneity (for a multivariate HR linear trend) of the associations of smoking with one molecular subtype versus the other molecular subtype (i.e., CIMP-low/negative vs. CIMP-high; MSS vs. MSI-high; *BRAF*-wildtype vs. *BRAF*-mutated; DNMT3B-negative vs. DNMT3B-positive).

[d] Models were adjusted for body mass index, family history of colorectal cancer in any first-degree relative, regular use of aspirin, physical activity level, alcohol consumption, total caloric intake, and red meat intake.

Table 6. Cumulative Pack-years of Smoking and Incident Colorectal Cancer Risk by Combined Molecular Subtypes[a] in the Nurses' Health Study (1980–2008) and the Health Professionals Follow-up Study (1986–2008)

	Never Smoker		Cumulative Pack-years of Smoking						P_{trend}[b]
			1–19		20–39		≥40		
	HR	95% CI	HR	95% CI	HR	95% CI	HR	95% CI	
CIMP/MSI Subtyping									
CIMP-low/negative									
MSS									
No.		340		227		161		137	
Age-adjusted	1.00	Referent	1.18	0.99, 1.40	1.20	0.99, 1.45	1.22	1.00, 1.49	0.05
Multivariate[c]	1.00	Referent	1.15	0.97, 1.37	1.16	0.96, 1.41	1.16	0.95, 1.43	0.15
MSI-high									
No.		19		10		8		6	
Age-adjusted	1.00	Referent	0.98	0.45, 2.11	1.14	0.49, 2.62	1.14	0.45, 2.88	0.72
Multivariate[c]	1.00	Referent	0.96	0.44, 2.07	1.08	0.47, 2.48	1.08	0.43, 2.75	0.82
CIMP-high									
MSS									
No.		29		11		7		14	
Age-adjusted	1.00	Referent	0.69	0.34, 1.39	0.60	0.26, 1.38	1.40	0.74, 2.67	0.42
Multivariate[c]	1.00	Referent	0.68	0.34, 1.37	0.58	0.25, 1.33	1.30	0.68, 2.48	0.56
MSI-high									
No.		41		22		28		42	
Age-adjusted	1.00	Referent	0.98	0.58, 1.65	1.90	1.17, 3.08	2.85	1.85, 4.40	<0.0001
Multivariate[c]	1.00	Referent	0.97	0.57, 1.63	1.82	1.12, 2.96	2.75	1.78, 4.26	<0.0001
CIMP/BRAF *Subtyping*									
CIMP-low/negative									
BRAF-wildtype									
No.		341		232		164		136	
Age-adjusted	1.00	Referent	1.20	1.02, 1.42	1.21	1.00, 1.46	1.23	1.00, 1.50	0.04
Multivariate[c]	1.00	Referent	1.17	0.99, 1.39	1.17	0.96, 1.41	1.17	0.95, 1.43	0.15
BRAF-mutated									
No.		22		8		6		10	
Age-adjusted	1.00	Referent	0.65	0.29, 1.48	0.81	0.33, 2.02	1.30	0.60, 2.81	0.49
Multivariate[c]	1.00	Referent	0.65	0.29, 1.47	0.81	0.32, 2.00	1.27	0.59, 2.75	0.53
CIMP-high									
BRAF-wildtype									
No.		28		12		13		20	
Age-adjusted	1.00	Referent	0.81	0.41, 1.60	1.14	0.59, 2.22	1.92	1.07, 3.42	0.02
Multivariate[c]	1.00	Referent	0.80	0.41, 1.59	1.10	0.56, 2.14	1.83	1.02, 3.27	0.03
BRAF-mutated									
No.		43		21		22		36	
Age-adjusted	1.00	Referent	0.87	0.52, 1.47	1.45	0.86, 2.43	2.44	1.56, 3.81	<0.0001
Multivariate[c]	1.00	Referent	0.86	0.51, 1.45	1.39	0.82, 2.33	2.32	1.48, 3.63	<0.0001
MSI/BRAF *Subtyping*									
MSS									
BRAF-wildtype									
No.		360		239		165		142	
Age-adjusted	1.00	Referent	1.16	0.99, 1.37	1.14	0.95, 1.38	1.20	0.99, 1.46	0.08
Multivariate[c]	1.00	Referent	1.13	0.96, 1.34	1.10	0.91, 1.33	1.14	0.93, 1.40	0.24

Table continues

Table 6. Continued

	Never Smoker		Cumulative Pack-years of Smoking						P_{trend}[b]
			1–19		20–39		≥40		
	HR	95% CI	HR	95% CI	HR	95% CI	HR	95% CI	
BRAF-mutated									
No.		36		14		9		17	
Age-adjusted	1.00	Referent	0.70	0.37, 1.30	0.70	0.34, 1.46	1.39	0.77, 2.50	0.35
Multivariate[c]	1.00	Referent	0.69	0.37, 1.28	0.68	0.33, 1.42	1.33	0.74, 2.40	0.42
MSI-high									
BRAF-wildtype									
No.		32		18		18		19	
Age-adjusted	1.00	Referent	1.04	0.58, 1.86	1.44	0.80, 2.57	1.86	1.04, 3.30	0.02
Multivariate[c]	1.00	Referent	1.03	0.57, 1.83	1.36	0.76, 2.44	1.79	1.01, 3.19	0.03
BRAF-mutated									
No.		30		16		19		31	
Age-adjusted	1.00	Referent	0.96	0.52, 1.76	1.82	1.02, 3.25	2.94	1.77, 4.87	<0.0001
Multivariate[c]	1.00	Referent	0.94	0.51, 1.72	1.74	0.97, 3.11	2.81	1.69, 4.68	<0.0001

Abbreviations: CI, confidence interval; CIMP, CpG island methylator phenotype; HR, hazard ratio; MSI, microsatellite instability; MSS, microsatellite stable.

[a] All models were stratified by calendar year of the questionnaire cycle, age, and sex.

[b] Based on the linear trend test by using the median value of each category.

[c] Models were adjusted for body mass index, family history of colorectal cancer in parent or sibling, regular use of aspirin, physical activity level, alcohol consumption, total caloric intake, and red meat intake.

find a possible preventive effect of smoking cessation on the development of the specific epigenotype (i.e., CIMP-high) of colorectal cancer.

Analyses of etiologic factors and molecular variation are important in epidemiologic research (66–68). One case-cohort study reported that duration of smoking cessation at study baseline was not associated with v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) oncogene mutation status, compared with never smokers (7, 69). To our knowledge, no previous study has prospectively examined the relation between duration of smoking cessation and colorectal cancer risk by tumor epigenetic features. Previous studies (26–31, 33, 34, 70–72) have shown positive associations between smoking and either MSI-high, CIMP-high, or *BRAF*-mutated cancer subtype. The case-control study by Samowitz et al. (27) attempted to subtype cancers using combined molecular subtypes, and reported that CIMP-high and *BRAF*-mutated cancer subtypes might be attributable to smoking. Caveats of that study (27) include the case-control design, and the use of methylation-specific polymerase chain reaction and the classic CIMP panel (12), which might not be as specific as the newer Weisenberger CIMP panel (18). The issue of tumor misclassification could be even more important when combined molecular subtyping is attempted. By using our large prospective cohort studies of men and women, and a validated MethyLight CIMP assay (20, 48), we were able to demonstrate that smoking was associated specifically with CIMP-high cancer risk and that the association between smoking and *BRAF*-mutated colorectal cancer appeared to be mediated by the well-known association between *BRAF* mutation and CIMP-high (18, 20, 27). Our data on smoking cessation also support the hypothesis that CIMP-high is the molecular subtype caused by smoking.

Our findings could have clinical implications in terms of personalized screening and prevention. With the emergence of assays that detect markers of DNA methylation in stool, specific screening tests might become available that could be targeted to smokers, as a particularly high-risk group for CIMP-high cancer. In addition, for other specific high-risk groups (e.g., older women) who are known to have greater susceptibility for CIMP-high cancer, smoking abstinence or cessation could prove to be a high-priority prevention strategy. Research on CIMP has been progressing (14, 16, 73–83), and besides smoking cessation, there might be effective prevention strategy for this unique cancer pathway.

There are several key strengths in our study. Firstly, the prospective design minimizes recall bias. Secondly, because we prospectively collected updated information on smoking every 2 years, we could assess the risk reduction by duration of smoking cessation as well as multiple smoking-related variables more precisely. Thirdly, we collected updated data on the known and many suspected risk factors for colorectal cancer from health professionals, who tend to report with high accuracy on medication use, allowing us to effectively control for potentially confounding variables. Finally, our tumor molecular analysis data enabled us to conduct integrative MPE research, which resulted in unique evidence for the association of duration of smoking cessation with a specific epigenotype of colorectal cancer.

Limitations of our study include the possibility of residual confounding including birth cohort effect, informative censoring and, in particular, a confounding effect of pack-years

on duration of cessation. To address the issue of pack-years smoked, we performed analysis stratified by cumulative pack-years. We could not obtain tumor paraffin blocks from all of the colorectal cancer cases. However, baseline features of participants without tumor analysis data did not differ materially from those with tumor analysis data. Our cohort represents a selected population, consisting of all health professionals, to maintain high compliance of questionnaire returns. Most of the participants are Caucasians. Therefore, the association of smoking cessation with cellular epigenetic instability in other occupational and other ethnic groups remains to be investigated. Results of sex-specific analysis need to be interpreted cautiously because of our limited statistical power in each sex stratum.

In summary, this prospective study suggests that smoking cessation could reduce the risk of the specific epigenotype, CIMP-high colorectal cancer. Our results provide not only insight into the colorectal carcinogenic mechanisms, but also yield further scientific support to the recommendation of smoking avoidance and cessation for the promotion of public health.

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