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ARTICLE

Smoking and Risk of Colorectal Cancer Sub-Classified by Tumor-Infiltrating T Cells

Tsuyoshi Hamada*, Jonathan A. Nowak*, Yohei Masugi*, David A. Drew, Mingyang Song, Yin Cao, Keisuke Kosumi, Kosuke Mima, Tyler S. Twombly, Li Liu, Yan Shi, Annacarolina da Silva, Mancang Gu, Wanwan Li, Katsuhiko Nosho, NaNa Keum, Marios Giannakis, Jeffrey A. Meyerhardt, Kana Wu, Molin Wang, Andrew T. Chan, Edward L. Giovannucci, Charles S. Fuchs†, Reiko Nishihara†, Xuehong Zhang†, Shuji Ogino†

See the Notes section for the full list of authors' affiliations.
Correspondence to: Shuji Ogino, MD, PhD, MS, Program in MPE Molecular Pathological Epidemiology, Brigham and Women's Hospital, 450 Brookline Ave., Room SM1036, Boston, MA 02215 (e-mail: shuji_ogino@dfci.harvard.edu).
*TH, JAN, and YM contributed equally as co-first authors.
†CSF, RN, XZ, and SO contributed equally as co-last authors.
Abbreviations: CI, confidence interval; CIMP, CpG island methylator phenotype; HPFS, Health Professionals Follow-up Study; HR, hazard ratio; METS, metabolic equivalent task score; MSI, microsatellite instability; NHS, Nurses' Health Study; TIL, tumor-infiltrating lymphocytes.

Abstract

Background: Evidence indicates not only carcinogenic effect of cigarette smoking but also its immunosuppressive effect. We hypothesized that the association of smoking with colorectal cancer risk might be stronger for tumors with lower anti-tumor adaptive immune response.

Methods: During follow-up of 134 981 participants (3 490 851 person-years) in the Nurses' Health Study and Health Professionals Follow-up Study, we documented 729 rectal and colon cancer cases with available data on T-cell densities in tumor microenvironment. Using the duplication-method Cox regression model, we examined a differential association of smoking status with risk of colorectal carcinoma subclassified by densities of *CD3*$^+$ cells, *CD8*$^+$ cells, CD45RO (*PTPRC*)$^+$ cells, or *FOXP3*$^+$ cells. All statistical tests were two-sided.

Results: The association of smoking status with colorectal cancer risk differed by *CD3*$^+$ cell density ($P_{heterogeneity} = .007$). Compared with never smokers, multivariable-adjusted hazard ratios for *CD3*$^+$ cell-low colorectal cancer were 1.38 (95% confidence interval = 1.09 to 1.75) in former smokers and 1.59 (95% confidence interval = 1.14 to 2.23) in current smokers ($P_{trend} = .002$, across smoking status categories). In contrast, smoking status was not associated with *CD3*$^+$ cell-high cancer risk ($P_{trend} = .52$). This differential association appeared consistent in strata of microsatellite instability, CpG island methylator phenotype, or *BRAF* mutation status. There was no statistically significant differential association according to densities of *CD8*$^+$ cells, CD45RO$^+$ cells, or *FOXP3*$^+$ cells ($P_{heterogeneity} > .04$, with adjusted α of 0.01).

Conclusions: Colorectal cancer risk increased by smoking was stronger for tumors with lower T-lymphocyte response, suggesting an interplay of smoking and immunity in colorectal carcinogenesis.

Cigarette smoking appears to be a modest risk factor for incidence of rectal and colon cancer (1–3). Colorectal carcinoma is a heterogeneous group of neoplasms with various combinations of molecular alterations and complex interactions with host cells in the tumor microenvironment (4–9). Epidemiological studies have shown that the association of smoking with

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colorectal cancer risk may be stronger for tumors characterized by high-degree CpG island methylator phenotype (CIMP), and high-level microsatellite instability (MSI) due to epigenetic silencing of *MLH1* gene (10–13). These findings provide evidence that smoking may induce epigenetic changes that can drive colorectal carcinogenesis (14,15).

The pivotal role of the host immunity in regulating neoplastic progression is gaining greater attention (16–21). MSI-high colorectal cancer with numerous frameshift mutations may produce abundant immunogenic peptides that can elicit intense immune response in the tumor microenvironment (22–24). One important component of immune response to tumor is lymphocytic infiltrate, which has been associated with prolonged patient survival in colorectal cancer (25–29). Accumulating evidence indicates that smoking can not only provoke systemic inflammation but also impair adaptive and innate immunity (30,31). In addition, experimental data suggest that smoking may suppress T cell-mediated tumor-specific immunity, potentially promoting tumor evolution (31–33). We therefore hypothesized that the association of smoking with colorectal cancer risk might be stronger for carcinomas with lower T cell density than for carcinomas with higher T cell density.

To test our hypothesis, we conducted a molecular pathological epidemiology study using integrated data on lifestyle factors, colorectal cancer incidence, and pathological immune response to tumor within two large prospective cohort studies. We examined differential associations of smoking with colorectal cancer risk according to densities of tumor-infiltrating T cells. The T cell co-receptor *CD3* helps to activate effector function of T cells, and we evaluated *CD3* expression as a marker of pan-T cells. We also evaluated specific T cell markers including *CD8* for cytotoxic T cells, CD45RO (an isoform of the *PTPRC* protein) for memory T cells, and *FOXP3* for regulatory T cells; the densities of these cells have been associated with high-level MSI and patient prognosis in colorectal cancer (20,26).

Methods

Study Population

We utilized two prospective cohort studies in the United States: the Nurses' Health Study (NHS, 121 701 women aged 30–55 years followed since 1976) and the Health Professionals Follow-up Study (HPFS, 51 529 men aged 40–75 years followed since 1986) (Table 1) (34). Participants have been sent questionnaires to report lifestyle factors, including smoking behavior and newly diagnosed diseases, every two years and to report dietary patterns every four years. At baseline, we excluded participants who did not report smoking status, did not return the initial food frequency questionnaire (in 1980 for the NHS and 1986 for the HPFS), left a large number of items blank (>10 of 61 items for the NHS and >70 of 131 items for the HPFS), or reported unreasonable food intakes (<600 or >3500 calories/day for women, and <800 or >4200 calories/day for men). We also excluded participants with a history of inflammatory bowel disease or cancer (except for nonmelanoma skin cancer). Participants were followed until diagnosis of colorectal cancer, death, or the end of follow-up (January 31, 2012 for the HPFS and June 1, 2012 for the NHS), whichever came first. The follow-up rate was more than 90% for each follow-up questionnaire in both cohorts.

Informed consent was obtained from all participants at enrollment. This study was approved by the institutional review boards at Harvard T.H. Chan School of Public Health and Brigham and Women's Hospital (Boston, MA).

Assessment of Smoking Behavior

Detailed information on smoking behavior was collected as reported previously (12,35). Current smoking status and daily cigarette consumption for smokers have been reported by participants on biennial questionnaires since 1980 (the NHS) and 1986 (the HPFS). On the baseline questionnaires (1976 in the NHS and 1986 in the HPFS), participants were asked to report the age at which they began and ceased smoking (if applicable), as well as the average daily consumption of cigarettes. We calculated the duration of smoking cessation and cumulative pack-years of smoking (average daily consumption of cigarette packs x the number of years smoked).

Ascertainment of Colorectal Cancer Cases

In both cohorts, colorectal cancer cases were identified based on biennial questionnaires. For nonrespondents with colorectal cancer, colorectal cancer cases and deaths were ascertained using various information sources, including family members, US Postal Service authorities, and the National Death Index. We included both colon and rectal carcinomas, based on the colorectal continuum model (36,37). Study physicians, blinded to exposure data, reviewed medical records of identified colorectal cancer cases to confirm the diagnosis and record tumor characteristics. We collected formalin-fixed, paraffin-embedded tissue blocks of surgically resected tumors from hospitals throughout the US. Demographic or clinical data did not differ substantially by availability of tumor tissue (Supplementary Table 1, available online) (38).

Analyses of Tumor Pathological, Immune, and Molecular Features

A single pathologist (SO), blinded to other data, reviewed hematoxylin and eosin-stained tissue sections, confirmed diagnosis of colorectal carcinoma, and recorded pathological features including four components of lymphocytic reaction (tumor-infiltrating lymphocytes, intratumoral periglandular reaction, peritumoral lymphocytic reaction, and Crohn's-like lymphoid reaction) (25). Each component was scored as negative/low, intermediate, or high. We constructed tissue microarrays for colorectal cancer cases with sufficient tissue materials, which included up to four cores from each case considering intratumor heterogeneity (39). We used the data on tumor-infiltrating T cells from our previous study (26). We measured densities (cells/mm^2) of *CD3*$^+$ cells, *CD8*$^+$ cells, CD45RO$^+$ cells, and *FOXP3*$^+$ cells in colorectal cancer tissue based on immunohistochemistry and image analysis using an automated scanning microscope and the Ariol image analysis system (Genetix, San Jose, CA) (Figure 1). We marked neoplastic epithelial areas to exclude nonneoplastic areas (eg, stroma, normal mucosa, and necrotic regions). We averaged the densities of each T cell subset within a patient and dichotomized the density by the median value of the total colorectal cancer population. DNA was extracted from archival formalin-fixed, paraffin-embedded tumor tissue blocks. Tumor status of MSI, CIMP, and *BRAF* mutation was determined as previously described (40,41).

Table 1. Age-standardized characteristics of participants according to smoking status in the Nurses' Health Study (NHS, 1980–2012) and the Health Professionals Follow-up Study (HPFS, 1986–2012)

	Women (NHS)			Men (HPFS)		
	Smoking status			Smoking status		
Characteristic*	Never	Former	Current	Never	Former	Current
Participants, person-years	5 69 176	5 14 352	1 95 743	2 44 818	2 22 206	30 985
Age, y	60.9 (11.5)	62.3 (11.0)	55.8 (10.1)	63.0 (11.3)	65.4 (10.9)	60.3 (10.0)
Family history of colorectal cancer	13.4%	13.8%	12.0%	12.5%	12.5%	12.0%
History of diabetes	7.4%	7.7%	6.3%	6.6%	8.2%	8.3%
Body mass index, kg/m^2	24.4 (4.7)	24.4 (4.6)	23.1 (4.1)	25.6 (3.4)	26.0 (3.3)	25.5 (3.4)
Postmenopause	76.4%	78.7%	78.4%	—	—	—
Menopausal hormone therapy	27.6%	29.1%	20.0%	—	—	—
History of colonoscopy/sigmoidoscopy	39.5%	42.6%	29.5%	53.8%	56.1%	43.4%
Multivitamin use	52.5%	53.6%	45.2%	44.4%	45.8%	39.2%
Regular use of aspirin	39.6%	41.3%	40.4%	45.5%	50.4%	45.2%
Regular use of other NSAIDs	17.1%	20.1%	14.4%	14.4%	17.2%	14.4%
Physical activity, METS-hours/week	16.5 (16.8)	17.3 (17.9)	14.1 (17.7)	26.8 (23.6)	25.4 (22.1)	19.7 (19.3)
Total calorie intake, kcal/day	1702 (443)	1669 (427)	1645 (457)	1984 (554)	1967 (549)	2013 (582)
Alcohol intake, g/day	3.8 (6.9)	7.3 (9.6)	9.0 (12.3)	8.0 (11.1)	13.5 (14.9)	17.0 (18.7)
Red and processed meat intake, servings/week	6.6 (3.7)	6.3 (3.4)	7.2 (3.8)	6.1 (4.3)	6.4 (4.4)	8.2 (5.0)
Calcium intake, mg/day	939 (357)	949 (349)	839 (335)	957 (375)	921 (368)	863 (364)
Folate intake, μg/day	429 (212)	433 (208)	381 (212)	550 (252)	544 (251)	483 (246)
Alternate Healthy Eating Index 2010†	46.1 (9.6)	47.5 (9.5)	43.5 (9.2)	48.6 (10.1)	48.4 (9.9)	43.6 (9.5)

*All variables other than age were standardized to age distribution of each cohort. Mean (standard deviation) was presented for continuous variables. HPFS = Health Professionals Follow-up Study; METS = metabolic equivalent task score; NHS = Nurses' Health Study; NSAIDs = nonsteroidal anti-inflammatory drugs.
†Without alcohol intake.

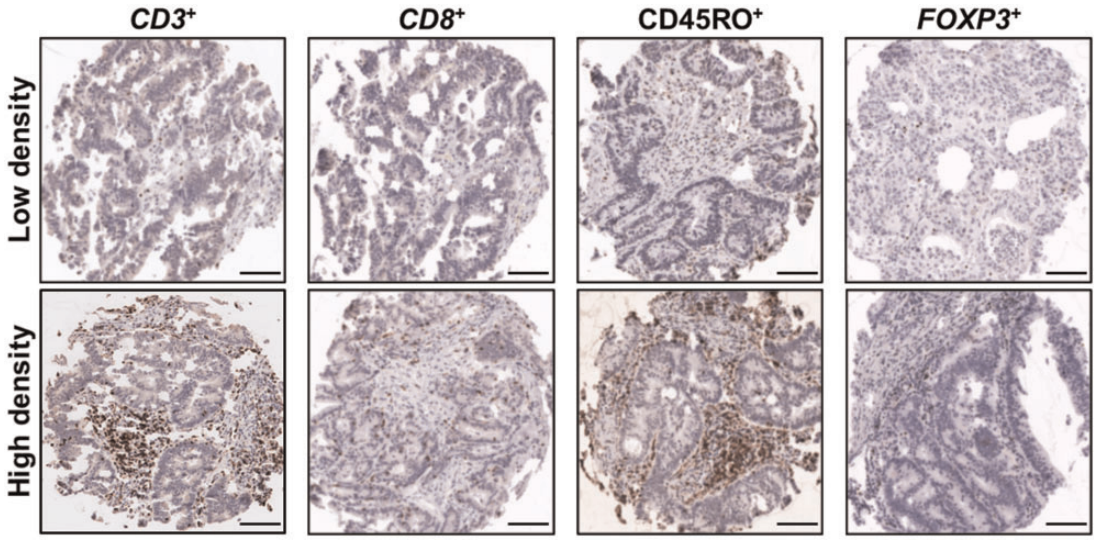

Figure 1. Tissue microarray images of *CD3*$^+$ cells, *CD8*$^+$ cells, CD45RO$^+$ cells, and *FOXP3*$^+$ cells in colorectal cancer. Upper and lower panels demonstrate tumors with low and high densities of T cell subsets, respectively. Scale bar = 100 μm.

Statistical Analysis

All statistical analyses were performed using SAS software (version 9.4, SAS Institute, Cary, NC), and all *P* values were two-sided. Our primary hypothesis testing was set to assess the heterogeneity between associations of smoking status (never, former, or current) with the risk of colorectal cancer subclassified by T cell densities (low vs high). We assessed the four T cell subsets (*CD3*$^+$ cells, *CD8*$^+$ cells, CD45RO$^+$ cells, and *FOXP3*$^+$ cells) and adjusted the α level to 0.01 ($\approx$ 0.05/4) based on Bonferroni correction. All other assessments including evaluation of individual hazard ratio (HR) estimates represented secondary analyses, and we used the adjusted α level of 0.01.

We used the Cox proportional hazards regression model to estimate HR of colorectal cancer incidence and the corresponding 95% confidence interval (CI). To assess the differential association of smoking status with incidence of colorectal cancer subclassified by T cell densities, we utilized the

duplication-method Cox regression model for competing risks (42). In analyses of a specific subtype of colorectal cancer, occurrence of other subtypes was treated as competing risk events. Using a likelihood ratio test, we examined the heterogeneity of subtype-specific associations and presented the statistical significance as a *P* value for heterogeneity ($P_{heterogeneity}$) (43). In the multivariable Cox model, we initially included the covariates described in Table 2 and conducted a backward elimination with a threshold *P* of .05 to select variables for the final model. Cases with missing data (<6.1% for all covariates) were assigned to the majority category or the median value of a given covariate to limit the degrees of freedom of the models. For cases with missing data on menopause status/menopausal hormone therapy (11.0%), we assigned a separate indicator variable. The models were stratified by sex (only for pooled analyses), age, and calendar year of questionnaire cycle. Colorectal cancer cases without available data on T cell density and participants who died without diagnosis of colorectal cancer were treated as censored cases at the time of cancer diagnosis and death, respectively. We treated all variables as time dependent to account for changes over time. To reduce intra-individual variation and consider long-term influences, we used the cumulative average for relevant variables, which was the mean of all available data prior to each questionnaire cycle. When the assumption of proportionality of hazards was verified by assessing a time-varying covariate (ie, the cross-product of smoking status and follow-up time), we observed evidence on violation of this assumption. However, the Schoenfeld residual plots supported the proportionality of hazards during most of the follow-up period (data not shown), and thus, we used the Cox regression model. To examine the association of smoking status with colorectal cancer risk by T cell density adjusted for tumor MSI, CIMP, or *BRAF* mutation status, we conducted meta-regression analyses for multiple tumor subtyping markers (44). We conducted tests of heterogeneity using the Q statistic and observed no statistically significant heterogeneity between the two cohorts ($P_{heterogeneity} > .13$) for the association of smoking status with colorectal cancer subclassified by T cell densities. We therefore combined the cohorts for further analyses to increase statistical power.

Results

Table 1 shows age-standardized characteristics of participants according to smoking status in the NHS and HPFS. During follow-up of 134 981 participants (3490 851 person-years), we documented 3066 colorectal cancer cases (1794 cases in the NHS and 1272 cases in the HPFS). Among these cases, there were 729 cases with available data on tumor-infiltrating T cells (463 cases [25.8%] in the NHS and 266 cases [20.9%] in the HPFS; Supplementary Table 1, available online). The distributions and correlation patterns by the two-sided Spearman correlation test of T cell subsets are shown in Supplementary Figure 1 and Supplementary Table 2, available online. Clinical, pathological, and molecular characteristics of colorectal cancer patients according to T cell densities are shown in Supplementary Table 3, available online. Smoking status was not associated with the risk of colorectal cancer overall in this population (Table 2).

In our primary hypothesis testing (Table 2), the association of smoking status with colorectal cancer risk differed by $CD3^+$ cell density ($P_{heterogeneity} = .007$; with the adjusted α level of 0.01). Compared with never smokers, former and current smokers were associated with a higher risk of $CD3^+$ cell-low colorectal cancer (multivariable HR = 1.38 [95% CI = 1.09 to 1.75] and 1.59 [95% CI = 1.14 to 2.23], respectively; $P_{trend} = .002$, across never, former, and current smokers). In contrast, smoking status was not associated with the risk of $CD3^+$ cell-high colorectal cancer ($P_{trend} = .52$); compared with never smokers, former and current smokers had multivariable HRs of 1.05 (95% CI = 0.83 to 1.31) and 0.78 (95% CI = 0.52 to 1.18), respectively. Among 690 cases with available data on $CD3^+$ cell densities, we evaluated four, three, two, and one tumor cores in tissue microarrays for 309 (44.8%), 64 (9.3%), 316 (45.8%), and 1 (0.1%) cases, respectively. Smoking status at the questionnaire immediately before colorectal cancer diagnosis was inversely associated with $CD3^+$ cell density ($P = .001$; Supplementary Table 4, available online), indicating the enrichment of $CD3^+$ cell-low cancers in current smokers. We did not observe statistically significant heterogeneity for associations of smoking status with tumor subtypes characterized by $CD8^+$ cells, $CD45RO^+$ cells, or $FOXP3^+$ cells ($P_{heterogeneity} > .04$, with adjusted α of 0.01). In secondary analyses, we examined the association of cumulative pack-years of smoking or duration of smoking cessation with colorectal cancer risk by densities of T cells (Table 3, and Supplementary Table 5, available online, respectively). Cumulative pack-years smoked were associated with a higher risk of $CD3^+$ cell-low colorectal cancer ($P_{trend} < .001$), but not with the risk of $CD3^+$ cell-high cancer ($P_{trend} = .68$). Smoking status was associated with the amount of alcohol consumption, which might influence the host immune system (45). However, we did not find a differential association of the amount of alcohol consumption with colorectal cancer risk by the density of T cells (data not shown).

The association of smoking with the risk of $CD3^+$ cell-low colorectal cancer appeared to be stronger than that with the risk of $CD3^+$ cell-high cancer, regardless of tumor MSI, CIMP, or *BRAF* mutation status (Table 4), but statistical power was limited. In meta-regression analyses (44), a stronger association of smoking with $CD3^+$ cell-low colorectal cancer compared with that with $CD3^+$ cell-high cancer appeared to be consistent after adjustment for tumor MSI, CIMP, or *BRAF* mutation status (Table 5).

When we examined a differential association of smoking status with incidence of colorectal cancer subclassified by level of lymphocytic reaction, the association of smoking status with colorectal cancer risk appeared to be stronger for tumors with higher-level Crohn's-like lymphoid reaction than for tumors with lower-level reaction (Supplementary Table 6, available online). However, this differential association was not observed after adjustment for tumor MSI status (Supplementary Table 7, available online).

Discussion

In this large prospective study with up to 30 years of follow-up, we found a stronger association of smoking with colorectal carcinomas with lower T cell density than with higher T cell density. This differential association appeared consistent regardless of tumor status of MSI, CIMP, or *BRAF* mutation. Our current study suggests that the carcinogenic effects of smoking may be, at least in part, mediated through local suppression of T cell-mediated immune response to colorectal tumor.

The promising results of trials of immune checkpoint inhibitors that target the *PDCD1* (programmed cell death 1, PD-1) or *CD274* (*PDCD1* ligand 1, PD-L1) protein have cast light on the modulation of T cell activity in the tumor microenvironment as a potential strategy for cancer treatment (46–48). Modulation of the immune system can also provide cancer

Table 2. Smoking status and colorectal cancer risk, overall and by T cell densities

Colorectal cancer subtype	Smoking status			P_{trend}*	$P_{heterogeneity}$†
	Never	Former	Current		
Person-years	16 03 868	1 438 649	448 334		
All colorectal cancer (n = 729)					
n	294	351	84		
Age-adjusted HR (95% CI)	1 (referent)	1.19 (1.02 to 1.39)	1.19 (0.93 to 1.53)	.04	
Multivariable HR (95% CI)‡	1 (referent)	1.16 (0.99 to 1.36)	1.11 (0.86 to 1.43)	.16	
CD3$^+$ cell density					
Low (n = 347)					.007
n	121	174	52		
Age-adjusted HR (95% CI)	1 (referent)	1.43 (1.13 to 1.80)	1.72 (1.23 to 2.40)	< .001	
Multivariable HR (95% CI)‡	1 (referent)	1.38 (1.09 to 1.75)	1.59 (1.14 to 2.23)	.002	
High (n = 343)					
n	152	162	29		
Age-adjusted HR (95% CI)	1 (referent)	1.07 (0.86 to 1.34)	0.84 (0.56 to 1.27)	.79	
Multivariable HR (95% CI)‡	1 (referent)	1.05 (0.83 to 1.31)	0.78 (0.52 to 1.18)	.52	
CD8$^+$ cell density					
Low (n = 339)					.05
n	127	164	48		
Age-adjusted HR (95% CI)	1 (referent)	1.28 (1.01 to 1.61)	1.60 (1.14 to 2.24)	.004	
Multivariable HR (95% CI)‡	1 (referent)	1.25 (0.98 to 1.58)	1.47 (1.04 to 2.07)	.01	
High (n = 338)					
n	145	163	30		
Age-adjusted HR (95% CI)	1 (referent)	1.14 (0.91 to 1.43)	0.85 (0.57 to 1.27)	.99	
Multivariable HR (95% CI)‡	1 (referent)	1.12 (0.89 to 1.41)	0.80 (0.54 to 1.20)	.75	
CD45RO$^+$ cell density					
Low (n = 352)					.99
n	139	177	36		
Age-adjusted HR (95% CI)	1 (referent)	1.24 (0.99 to 1.56)	1.11 (0.77 to 1.62)	.18	
Multivariable HR (95% CI)‡	1 (referent)	1.21 (0.96 to 1.51)	1.03 (0.71 to 1.50)	.37	
High (n = 348)					
n	144	161	43		
Age-adjusted HR (95% CI)	1 (referent)	1.14 (0.91 to 1.43)	1.21 (0.86 to 1.72)	.18	
Multivariable HR (95% CI)‡	1 (referent)	1.11 (0.88 to 1.40)	1.13 (0.80 to 1.60)	.36	
FOXP3$^+$ cell density					
Low (n = 332)					.30
n	134	150	48		
Age-adjusted HR (95% CI)	1 (referent)	1.11 (0.88 to 1.41)	1.47 (1.05 to 2.06)	.04	
Multivariable HR (95% CI)‡	1 (referent)	1.07 (0.84 to 1.36)	1.34 (0.96 to 1.89)	.12	
High (n = 333)					
n	134	173	26		
Age-adjusted HR (95% CI)	1 (referent)	1.31 (1.04 to 1.64)	0.82 (0.54 to 1.26)	.56	
Multivariable HR (95% CI)‡	1 (referent)	1.27 (1.01 to 1.59)	0.75 (0.49 to 1.16)	.93	

*P_{trend} was calculated using a two-sided linear tend test and ordinal categories of smoking status (never, former, and current). CI = confidence interval; HR = hazard ratio.

†$P_{heterogeneity}$ was calculated using a two-sided likelihood ratio test for the heterogeneity between subtype-specific multivariable association measures.

‡Adjusted for family history of colorectal cancer (present vs absent), history of diabetes (present vs absent), body mass index (continuous, a linear term and a squared term), history of colonoscopy/sigmoidoscopy (present vs absent), multivitamin use (regular use vs non-use), aspirin use (regular use vs non-use), use of other nonsteroidal anti-inflammatory drugs (regular use vs non-use), physical activity (continuous, a linear term and a squared term), total calorie intake (continuous, a linear term and a squared term), alcohol intake (continuous, a linear term and a squared term), red and processed meat intake (continuous, a linear term and a squared term), calcium intake (continuous, a linear term and a squared term), folate intake (continuous, a linear term and a squared term), and Alternate Healthy Eating Index 2010 (continuous, a linear term and a squared term; without alcohol). For women, we additionally adjusted for menopause status/menopausal hormone therapy (premenopause vs postmenopause with never, past, or current use of menopausal hormone therapy). A backward elimination with a threshold *P* of .05 was used to select variables for the final model. The final model included family history of colorectal cancer, body mass index (linear term), history of colonoscopy/sigmoidoscopy, aspirin use, and calcium intake (squared term). The Cox models were stratified by age, calendar year of questionnaire cycle, and sex/cohort.

immunoprevention strategies (49,50). Evidence indicates that various dietary, lifestyle, and environmental factors may stimulate or suppress anti-tumor immune response during tumor development (51–53). Therefore, integrated investigations of environmental exposures, tumor molecular features, and immune parameters are of increasing importance (51,54). A better understanding of the etiologies of smoking-related colorectal carcinogenesis in the context of host-tumor interactions would enhance the efficacy of immunoprevention strategies (52,55,56).

The carcinogenic effects of smoking have been investigated in various tumor types including colorectal cancer (1,2). Cigarette smoke contains thousands of cancer-causing chemicals that induce DNA damage (14,15,57,58). Host immune

Table 3. Cumulative pack-years of smoking and colorectal cancer risk, overall and by T cell densities

Colorectal cancer subtype	Cumulative pack-years of smoking				P_{trend}*	$P_{heterogeneity}$†
	0	1–19	20–39	≥ 40		
Person-years	16 03 868	9 40 185	5 58 187	3 72 396		
All colorectal cancer (n = 716)						
n	294	163	126	133		
Age-adjusted HR (95% CI)	1 (referent)	1.03 (0.85 to 1.25)	1.16 (0.94 to 1.43)	1.41 (1.15 to 1.74)	<.001	—
Multivariable HR (95% CI)‡	1 (referent)	1.02 (0.84 to 1.24)	1.12 (0.90 to 1.38)	1.31 (1.05 to 1.62)	.01	—
CD3$^+$ cell density						
Low (n = 344)						.03
n	121	84	65	74		
Age-adjusted HR (95% CI)	1 (referent)	1.29 (0.98 to 1.71)	1.42 (1.05 to 1.92)	1.89 (1.41 to 2.54)	<.001	
Multivariable HR (95% CI)‡	1 (referent)	1.27 (0.96 to 1.68)	1.36 (1.00 to 1.85)	1.75 (1.30 to 2.36)	<.001	
High (n = 335)						
n	152	73	56	54		
Age-adjusted HR (95% CI)	1 (referent)	0.89 (0.67 to 1.18)	1.04 (0.76 to 1.41)	1.13 (0.82 to 1.55)	.36	
Multivariable HR (95% CI)‡	1 (referent)	0.89 (0.67 to 1.18)	1.00 (0.73 to 1.36)	1.05 (0.76 to 1.44)	.68	
CD8$^+$ cell density						
Low (n = 338)						.11
n	127	81	60	70		
Age-adjusted HR (95% CI)	1 (referent)	1.19 (0.90 to 1.57)	1.31 (0.96 to 1.79)	1.67 (1.24 to 2.24)	<.001	
Multivariable HR (95% CI)‡	1 (referent)	1.18 (0.89 to 1.56)	1.26 (0.93 to 1.73)	1.55 (1.14 to 2.09)	.005	
High (n = 328)						
n	145	75	59	49		
Age-adjusted HR (95% CI)	1 (referent)	0.95 (0.71 to 1.25)	1.09 (0.81 to 1.49)	1.11 (0.80 to 1.55)	.39	
Multivariable HR (95% CI)‡	1 (referent)	0.94 (0.71 to 1.25)	1.06 (0.78 to 1.44)	1.05 (0.75 to 1.46)	.65	
CD45RO$^+$ cell density						
Low (n = 348)						.15
n	139	79	54	76		
Age-adjusted HR (95% CI)	1 (referent)	1.05 (0.79 to 1.39)	1.04 (0.76 to 1.43)	1.70 (1.28 to 2.26)	<.001	
Multivariable HR (95% CI)‡	1 (referent)	1.04 (0.78 to 1.37)	1.00 (0.72 to 1.37)	1.57 (1.18 to 2.10)	.005	
High (n = 340)						
n	144	78	67	51		
Age-adjusted HR (95% CI)	1 (referent)	1.01 (0.76 to 1.33)	1.29 (0.96 to 1.72)	1.11 (0.80 to 1.53)	.24	
Multivariable HR (95% CI)‡	1 (referent)	1.00 (0.76 to 1.33)	1.24 (0.92 to 1.66)	1.03 (0.75 to 1.43)	.49	
FOXP3$^+$ cell density						
Low (n = 329)						.03
n	134	59	67	69		
Age-adjusted HR (95% CI)	1 (referent)	0.82 (0.60 to 1.11)	1.33 (0.99 to 1.79)	1.63 (1.22 to 2.19)	<.001	
Multivariable HR (95% CI)‡	1 (referent)	0.80 (0.59 to 1.09)	1.27 (0.94 to 1.71)	1.49 (1.10 to 2.00)	.001	
High (n = 323)						
n	134	88	51	50		
Age-adjusted HR (95% CI)	1 (referent)	1.23 (0.94 to 1.61)	1.07 (0.77 to 1.48)	1.16 (0.84 to 1.61)	.52	
Multivariable HR (95% CI)‡	1 (referent)	1.22 (0.93 to 1.60)	1.02 (0.73 to 1.41)	1.07 (0.76 to 1.49)	.93	

*P_{trend} was calculated using a two-sided linear tend test and the median value of each category of cumulative pack-years of smoking (continuous). CI = confidence interval; HR = hazard ratio.

†$P_{heterogeneity}$ was calculated using a two-sided likelihood ratio test for the heterogeneity between subtype-specific multivariable association measures.

‡Adjusted for the same set of covariates as Table 2. The Cox models were stratified by age, calendar year of questionnaire cycle, and sex/cohort.

response plays a vital role in eliminating aberrant cells and inhibiting tumor formation (51), and the suppressive effects of smoking on adaptive and innate immune response have been proposed as alternative mechanisms of smoking-related carcinogenesis (31–33). Smoking suppresses the function of cells associated with innate immunity, including dendritic cells, NK cells, and macrophages, thereby suppressing Th1 cell activity and hampering the immunosurveillance mechanisms (30–33). Nicotine, a major component of cigarette smoke, acts as an agonist of nicotinic acetylcholine receptors and may particularly contribute to the suppression of immunosurveillance (30,32). Nicotine may not only activate signaling pathways such as the RAS-RAF-*MAP2K* (MEK)-*MAPK1* (ERK) pathway and *JAK2-STAT3* pathway, but also compromise anti-tumor immunosurveillance through suppression of NK cells and dendritic cells (32,59,60). Experimental data also indicate that exposure to cigarette smoke extract directly impairs T cell function via a reduction in cell proliferation and induction of cellular apoptosis (30,61) and specifically affects levels of cytotoxic, regulatory, and helper T cells as well as B cells (31). Furthermore, human population data also point to the immunosuppressive effects associated with smoking, including an increase in circulating regulatory T cells and a reduction in a wide spectrum of immune markers (62,63). Taking these facts into account, long-term exposure to smoking may induce immunosuppressive conditions in the tumor microenvironment, thereby assisting the evasion of immune mechanisms by tumor cells. Our study supports the possibility that smoking may impair the T cell-mediated

Table 4. Smoking status and colorectal cancer risk by *CD3*$^+$ cell densities in strata of tumor microsatellite instability (MSI), CpG island methylator phenotype (CIMP), or *BRAF* mutation status

Colorectal cancer subtype	Smoking status			P_{trend}*
	Never	Former	Current	
Non-MSI-high				
CD3$^+$ cell density				
Low (n = 292)				
n	101	149	42	
Age-adjusted HR (95% CI)	1 (referent)	1.46 (1.13 to 1.89)	1.63 (1.13 to 2.35)	.001
Multivariable HR (95% CI)†	1 (referent)	1.42 (1.10 to 1.83)	1.48 (1.02 to 2.15)	.008
High (n = 276)				
n	122	133	21	
Age-adjusted HR (95% CI)	1 (referent)	1.08 (0.85 to 1.39)	0.74 (0.46 to 1.19)	.58
Multivariable HR (95% CI)†	1 (referent)	1.06 (0.82 to 1.36)	0.68 (0.43 to 1.10)	.36
MSI-high				
CD3$^+$ cell density				
Low (n = 53)				
n	19	24	10	
Age-adjusted HR (95% CI)	1 (referent)	1.25 (0.68 to 2.30)	2.41 (1.10 to 5.27)	.05
Multivariable HR (95% CI)†	1 (referent)	1.28 (0.69 to 2.35)	2.48 (1.12 to 5.49)	.04
High (n = 55)				
n	23	24	8	
Age-adjusted HR (95% CI)	1 (referent)	1.10 (0.62 to 1.95)	1.73 (0.76 to 3.96)	.27
Multivariable HR (95% CI)†	1 (referent)	1.10 (0.61 to 1.96)	1.76 (0.76 to 4.05)	.27
CIMP-low/negative				
CD3$^+$ cell density				
Low (n = 290)				
n	103	150	37	
Age-adjusted HR (95% CI)	1 (referent)	1.46 (1.14 to 1.88)	1.40 (0.96 to 2.06)	.009
Multivariable HR (95% CI)†	1 (referent)	1.42 (1.10 to 1.83)	1.29 (0.88 to 1.90)	.04
High (n = 280)				
n	119	137	24	
Age-adjusted HR (95% CI)	1 (referent)	1.15 (0.90 to 1.48)	0.88 (0.56 to 1.38)	.85
Multivariable HR (95% CI)†	1 (referent)	1.12 (0.87 to 1.44)	0.81 (0.52 to 1.27)	.82
CIMP-high				
CD3$^+$ cell density				
Low (n = 54)				
n	17	23	14	
Age-adjusted HR (95% CI)	1 (referent)	1.26 (0.67 to 2.36)	3.95 (1.91 to 8.15)	.001
Multivariable HR (95% CI)†	1 (referent)	1.25 (0.66 to 2.36)	3.94 (1.88 to 8.26)	.002
High (n = 53)				
n	27	22	4	
Age-adjusted HR (95% CI)	1 (referent)	0.82 (0.47 to 1.45)	0.73 (0.25 to 2.11)	.43
Multivariable HR (95% CI)†	1 (referent)	0.82 (0.46 to 1.45)	0.73 (0.25 to 2.12)	.43
BRAF wild-type				
CD3$^+$ cell density				
Low (n = 291)				
n	99	150	42	
Age-adjusted HR (95% CI)	1 (referent)	1.50 (1.16 to 1.93)	1.70 (1.18 to 2.46)	<.001
Multivariable HR (95% CI)†	1 (referent)	1.46 (1.13 to 1.89)	1.57 (1.08 to 2.28)	.003
High (n = 289)				
n	128	137	24	
Age-adjusted HR (95% CI)	1 (referent)	1.07 (0.84 to 1.36)	0.85 (0.55 to 1.33)	.84
Multivariable HR (95% CI)†	1 (referent)	1.05 (0.82 to 1.34)	0.79 (0.50 to 1.23)	.57
BRAF mutant				
CD3$^+$ cell density				
Low (n = 51)				
n	18	23	10	
Age-adjusted HR (95% CI)	1 (referent)	1.29 (0.69 to 2.40)	2.27 (1.03 to 4.98)	.06
Multivariable HR (95% CI)†	1 (referent)	1.25 (0.67 to 2.34)	2.15 (0.97 to 4.80)	.08
High (n = 48)				
n	20	23	5	
Age-adjusted HR (95% CI)	1 (referent)	1.17 (0.64 to 2.14)	0.95 (0.35 to 2.62)	.86
Multivariable HR (95% CI)†	1 (referent)	1.14 (0.62 to 2.09)	0.93 (0.33 to 2.57)	.93

*P_{trend} was calculated using a two-sided linear tend test and ordinal categories of smoking status (never, former, and current). CI = confidence interval; CIMP = CpG island methylator phenotype; HR = hazard ratio; MSI = microsatellite instability.

†Adjusted for the same set of covariates as Table 2. The Cox models were stratified by age, calendar year of questionnaire cycle, and sex/cohort.

Table 5. Meta-regression analyses to assess the association of smoking status with colorectal cancer risk by *CD3*$^+$ cell densities, adjusted for tumor microsatellite instability (MSI), CpG Island methylator phenotype (CIMP), or *BRAF* mutation status

	Smoking status			
Colorectal cancer subtype	Never (n = 265)	Former (n = 330)	Current (n = 81)	P_{trend}*
CD3$^+$ cell-low (vs *CD3*$^+$ cell-high), ratio of multivariable HRs (95% CI)†	1 (referent)	1.31 (0.94 to 1.82)	1.98 (1.16 to 3.38)	.01
MSI-high (vs non-MSI-high), ratio of multivariable HRs (95% CI)†	1 (referent)	0.97 (0.61 to 1.53)	2.03 (1.06 to 3.88)	.12
CD3$^+$ cell-low (vs *CD3*$^+$ cell-high), ratio of multivariable HRs (95% CI)†	1 (referent)	1.30 (0.93 to 1.81)	1.97 (1.15 to 3.37)	.01
CIMP-high (vs CIMP-low/negative), ratio of multivariable HRs (95% CI)†	1 (referent)	0.80 (0.50 to 1.26)	2.01 (1.02 to 3.96)	.28
CD3$^+$ cell-low (vs *CD3*$^+$ cell-high), ratio of multivariable HRs (95% CI)†	1 (referent)	1.34 (0.97 to 1.87)	2.05 (1.21 to 3.49)	.007
BRAF mutant (vs *BRAF* wild-type), ratio of multivariable HRs (95% CI)†	1 (referent)	0.97 (0.60 to 1.55)	1.29 (0.65 to 2.58)	.60

*P_{trend} was calculated using a two-sided linear tend test and ordinal categories of smoking status (never, former, and current). CI = confidence interval; CIMP = CpG island methylator phenotype; HR = hazard ratio; MSI = microsatellite instability.
†Multivariable HRs for colorectal cancer jointly classified by the density of *CD3*$^+$ cells, and tumor MSI, CIMP, or *BRAF* mutation status were estimated using the Cox models adjusted for the same set of covariates as Table 2. The Cox models were stratified by age, calendar year of questionnaire cycle, and sex/cohort. We then modeled subtype-specific HRs in meta-regression analyses.

immune response, thereby assisting the development of colorectal carcinoma.

Evidence indicates that smoking may increase the risk of MSI-high colorectal cancer, which is characterized by an intense immune response to the tumor (10–13,22–24,47). This could explain our findings of a higher risk of colorectal cancer characterized by high-level Crohn's-like lymphoid reaction associated with current smoking before adjustment for tumor MSI status. This differential association was no longer observed after adjustment for tumor MSI status. In contrast, our study found a seemingly counterintuitive association of smoking with an increased risk of *CD3*$^+$ cell-low cancer. However, our findings may suggest the following explanation. Without the immunosuppressive effects of smoking, a fraction of MSI-high tumors may be eliminated by the intense immune response characteristic of MSI-high tumors. Smoking may suppress this immune response, which leads to an increased risk of MSI-high cancers in smokers. Therefore, our findings of the stronger association of smoking with *CD3*$^+$ cell-low colorectal cancer provide evidence for the mediation of the carcinogenic activity of smoking through impairment of T cell-mediated anti-tumor immunity.

The current study has notable strengths. Owing to the biennial collection of data on lifestyle factors for more than 30 years of follow-up, we could evaluate the long-term effect of smoking on colorectal carcinogenesis without substantial recall bias while adjusting for potential confounders. Furthermore, we utilized the molecular pathological epidemiology approach to integrate data on lifestyle factors, cancer incidence, and tumor molecular and immune characteristics (51,54). The current study was based on the assessment of local T cell infiltrates in the colorectal cancer tissue rather than plasma immune biomarkers (62), thereby providing insights into the complex interactions between exposures, host factors, and neoplastic cells in the microenvironment.

The current study has limitations. First, there was the possibility of unmeasured confounding. Second, we could not obtain tumor tissue samples from all colorectal cancer cases. However, demographic or clinical characteristics did not differ substantially between colorectal cancer patients with and without tumor tissue data (38). Third, we evaluated up to four tumor cores in a tissue microarray for ease case considering the intra-tumor heterogeneity of T cell distributions, but this approach might still lead to a bias because of sampling of the cores. Nonetheless, this misclassification would most likely skew our findings toward the null association. Finally, our study was based on selected populations; most participants were non-Hispanic health professionals. Hence, our findings need to be validated in independent populations.

In conclusion, we have shown that the association of the risk of colorectal cancer with smoking is more pronounced for carcinoma subtype with lower quantities of *CD3*$^+$ cells than subtype with greater amounts of *CD3*$^+$ cells. Our study highlights the potential role of suppression of T cell-mediated antitumor immunity in mediating the effect of smoking on colorectal carcinogenesis.

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Notes

Affiliations of authors: Department of Oncologic Pathology (TH, YM, KK, TST, LL, YS, AdS, MGu, WL, RN, SO) and Department of Medical Oncology, (KM, MGi, JAM), Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA; Program in Molecular Pathological Epidemiology, Department of Pathology (JAN, RN, SO), Department of Medicine (MGi), and Channing Division of Network Medicine, Department of Medicine (KW, MW, ATC, ELG, XZ), Brigham and Women's Hospital and Harvard Medical School, Boston, MA; Clinical and Translational Epidemiology Unit, and Division of Gastroenterology, Massachusetts General Hospital and Harvard Medical School, Boston, MA (DAD, MS, YC, ATC); Department of Nutrition (MS, YC, LL, NK, KW, ELG, RN), Department of Epidemiology (KW, MW, ELG, RN, SO), and Department of Biostatistics (MW, RN), Harvard T.H. Chan School of Public Health, Boston, MA; Division of Public Health Sciences, Department of Surgery, Washington University School of Medicine, St. Louis, MO (YC); Department of Epidemiology and Biostatistics, and the Ministry of Education Key Lab of Environment and Health, School of Public Health, Huazhong University of Science and Technology, Hubei, P.R. China (LL); Department of Medical Oncology, Chinese PLA General Hospital, Beijing, P.R. China (YS); College of Pharmacy, Zhejiang Chinese Medical University, Zhejiang, P.R. China (MGu); Department of Gastroenterology, Rheumatology, and Clinical Immunology, Sapporo Medical University School of Medicine, Sapporo, Japan (KN); Department of Food Science and Biotechnology, Dongguk University, Goyang, the Republic of Korea (NK); Broad Institute of MIT and Harvard, Cambridge, MA (MGi, SO); Yale Cancer Center, New Haven, CT (CSF); Department of Medicine, Yale School of Medicine, New Haven, CT (CSF); Smilow Cancer Hospital, New Haven, CT (CSF).

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