Research Article

Cancer Immunology Research

Fusobacterium nucleatum in Colorectal Cancer Relates to Immune Response Differentially by Tumor Microsatellite Instability Status

Tsuyoshi Hamada[1], Xuehong Zhang[2], Kosuke Mima[3], Susan Bullman[3,4], Yasutaka Sukawa[3], Jonathan A. Nowak[5], Keisuke Kosumi[1], Yohei Masugi[1], Tyler S. Twombly[1], Yin Cao[6,7,8,9], Mingyang Song[6,7,8], Li Liu[1,8,10], Annacarolina da Silva[1], Yan Shi[1,11], Mancang Gu[1,12], Wanwan Li[1], Hideo Koh[1], Katsuhiko Nosho[13], Kentaro Inamura[14], NaNa Keum[8,15], Kana Wu[2,8,16], Jeffrey A. Meyerhardt[3], Aleksandar D. Kostic[4,17], Curtis Huttenhower[4,18], Wendy S. Garrett[3,4,19], Matthew Meyerson[3,4], Edward L. Giovannucci[2,8,16], Andrew T. Chan[2,6,7], Charles S. Fuchs[20,21,22], Reiko Nishihara[1,5,8,16,18], Marios Giannakis[3,4,23], and Shuji Ogino[1,4,5,16]

Abstract

The presence of *Fusobacterium nucleatum* (*F. nucleatum*) in colorectal carcinoma tissue has been associated with microsatellite instability (MSI), lower-level T-cell infiltrates, and poor clinical outcomes. Considering differences in the tumor–immune microenvironment between MSI-high and non–MSI-high carcinomas, we hypothesized that the association of *F. nucleatum* with immune response might differ by tumor MSI status. Using samples from 1,041 rectal and colon cancer patients within the Nurses' Health Study and Health Professionals Follow-up Study, we measured *F. nucleatum* DNA in tumor tissue by a quantitative polymerase chain reaction assay. Multivariable logistic regression models were used to examine the association between *F. nucleatum* status and histopathologic lymphocytic reactions or density of $CD3^+$ cells, $CD8^+$ cells, CD45RO $(PTPRC)^+$ cells, or $FOXP3^+$ cells in strata of tumor MSI status. We adjusted for potential confounders, including CpG island methylator phenotype; LINE-1 methylation; and *KRAS*, *BRAF*, and *PIK3CA* mutations. The association of *F. nucleatum* with tumor-infiltrating lymphocytes (TIL) and intratumoral periglandular reaction differed by tumor MSI status ($P_{interaction} = 0.002$). The presence of *F. nucleatum* was negatively associated with TIL in MSI-high tumors [multivariable odds ratio (OR), 0.45; 95% confidence interval (CI), 0.22–0.92], but positively associated with TIL in non–MSI-high tumors (multivariable OR 1.91; 95% CI, 1.12–3.25). No significant differential association was observed for peritumoral lymphocytic reaction, Crohn-like lymphoid reaction, or T-cell densities. In conclusion, the association of *F. nucleatum* with immune response to colorectal carcinoma differs by tumor MSI status, suggesting that *F. nucleatum* and MSI status interact to affect antitumor immune reactions. *Cancer Immunol Res; 6(11); 1327–36. ©2018 AACR.*

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[1]Department of Oncologic Pathology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts. [2]Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts. [3]Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts. [4]Broad Institute of MIT and Harvard, Cambridge, Massachusetts. [5]Program in MPE Molecular Pathological Epidemiology, Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts. [6]Clinical and Translational Epidemiology Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts. [7]Division of Gastroenterology, Massachusetts General Hospital, Boston, Massachusetts. [8]Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, Massachusetts. [9]Division of Public Health Sciences, Department of Surgery, Washington University School of Medicine, St. Louis, Missouri. [10]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. [11]Department of Medical Oncology, Chinese PLA General Hospital, Beijing, P.R. China. [12]College of Pharmacy, Zhejiang Chinese Medical University, Zhejiang, P.R. China. [13]Department of Gastroenterology, Rheumatology, and Clinical Immunology, Sapporo Medical University School of Medicine, Sapporo, Japan. [14]Division of Pathology, The Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan. [15]Department of Food Science and Biotechnology, Dongguk University, Goyang, the Republic of Korea. [16]Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, Massachusetts. [17]Research Division, Joslin Diabetes Center, Boston, Massachusetts. [18]Department of Biostatistics, Harvard T.H. Chan School of Public Health, Boston, Massachusetts. [19]Department of Immunology and Infectious Diseases, Harvard T.H. Chan School of Public Health, Boston, Massachusetts. [20]Yale Cancer Center, New Haven, Connecticut. [21]Department of Medicine, Yale School of Medicine, New Haven, Connecticut. [22]Smilow Cancer Hospital, New Haven, Connecticut. [23]Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts.

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T. Hamada, X. Zhang, K. Mima, and S. Bullman contributed equally as co-first authors. C.S. Fuchs, R. Nishihara, M. Giannakis, and S. Ogino contributed equally as co-last authors.

Corresponding Authors: Shuji Ogino, Brigham and Women's Hospital, Boston, MA 02215. Phone: 617-582-9526; Fax: 617-632-8558; E-mail: shuji_ogino@dfci.harvard.edu; and Marios Giannakis, Department of Medical Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue, Room D1220, Boston, MA 02215. Phone: 617-582-7263; Fax: 617-632-5370; E-mail: marios_giannakis@dfci.harvard.edu

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Introduction

Accumulating evidence continues to illustrate the importance of the host immune system in regulating the evolution and progression of neoplastic diseases (1, 2). The colorectum is rich in microbes, and the gut microbiome plays a role in regulating local and systemic immune status (3). *Fusobacterium nucleatum* (*F. nucleatum*) has emerged as a potentially influential bacterium implicated in colorectal carcinogenesis (4–7). Metagenomic and clinical studies have highlighted the enrichment of *F. nucleatum* in colorectal cancer tissue relative to adjacent normal epithelium (5–9), and its persistence in metastatic lesions (10). Experimental evidence indicates that *F. nucleatum* can elicit inflammatory reactions, impair T-cell–mediated antitumor immune response, and promote tumor cell proliferation (11–15). Studies have also demonstrated the association of *F. nucleatum* in colorectal cancer tissue with proximal tumor location, microsatellite instability (MSI), CpG island methylator phenotype (CIMP), lower levels of tumor-infiltrating $CD3^+$ cells, and poor patient prognosis (8, 16–19).

Colorectal cancer consists of a heterogeneous collection of neoplasms that evolves through stepwise accumulation of genetic and epigenetic aberrations, a process influenced by environmental exposures, the microbiota, and host factors (20–22). MSI-high colorectal carcinomas typically contain numerous frameshift mutations and generate immunogenic peptides ("neoantigens") due to mismatch-repair deficiency, which encourage the antitumor immune response and lead to more favorable prognosis and better response to immunotherapies (23–28). In fact, the FDA approved the use of anti-PDCD1 (programmed cell death 1, PD-1) antibodies pembrolizumab and nivolumab for colorectal cancer with high-level MSI or mismatch-repair deficiency. Considering the differences in the immune microenvironment between MSI-high and non–MSI-high tumors, we hypothesized that the association of *F. nucleatum* in colorectal carcinoma tissue with immune response might differ by tumor MSI status.

To test this hypothesis, we utilized a molecular pathological epidemiology (MPE) database of more than 1,000 colorectal carcinoma cases in two U.S. prospective cohort studies, with data on clinical, pathologic, and tumor molecular characteristics. This comprehensive data set enabled us to assess whether the association of *F. nucleatum* in colorectal cancer tissue with immune response to tumor might differ by MSI status while adjusting for a number of potential confounders.

Materials and Methods

Study population

We utilized two prospective cohort studies in the United States, the Nurses' Health Study (NHS, 121,701 women ages 30–55 years followed since 1976) and the Health Professionals Follow-up Study (HPFS, 51,529 men ages 40–75 years followed since 1986). Every two years, participants have been sent follow-up questionnaires to report lifestyle factors and newly diagnosed diseases including colorectal cancer. The follow-up rate was >90% for each follow-up questionnaire in both cohort studies. In both cohorts, the National Death Index was used to ascertain deaths of participants and identify unreported lethal colorectal cancer cases. Study physicians, blinded to exposure data, reviewed medical records of identified colorectal cancer cases to confirm the disease diagnosis and to collect data on clinical characteristics (e.g., tumor location and disease stage).

Among participants diagnosed with colorectal cancer up to 2012, we analyzed 1,041 cases with available data on *F. nucleatum* and immune response in tumor tissue samples. We included both colon and rectal carcinomas, based on the colorectal continuum model (29, 30). We excluded patients who had been preoperatively treated. We collected formalin-fixed paraffin-embedded (FFPE) tumor tissue blocks from hospitals throughout the United States where colorectal cancer patients had undergone surgical resection. A single pathologist (S. Ogino), blinded to other data, reviewed hematoxylin and eosin–stained tissue sections from all collected blocks and recorded pathologic features. Tumor differentiation was categorized into well/moderate versus poor (>50% vs. $\leq$50% gland formation, respectively).

Written informed consent was obtained from all participants at enrollment in the NHS and HPFS. This study was conducted in accordance with the Declaration of Helsinki, and after approval by the institutional review boards at Harvard T.H. Chan School of Public Health and Brigham and Women's Hospital (Boston, MA).

Quantitative PCR

DNA was extracted from colorectal cancer tissue in archival FFPE tissue sections using QIAamp DNA FFPE Tissue Kit (Qiagen). As previously described and validated (8), we performed a quantitative PCR assay to measure the amount of *F. nucleatum* DNA in the tumor using custom TaqMan primer/probe sets (Applied Biosystems) for the nusG gene of *F. nucleatum* and the reference human gene *SLCO2A1*. Amplification and detection of DNA were performed using the StepOnePlus Real-Time PCR Systems (Applied Biosystems). Amounts of intratumoral *F. nucleatum* DNA in each specimen were calculated as a relative value normalized to amount of *SLCO2A1* using the $2^{-\Delta Ct}$ method [where ΔCt (cycle threshold) = "the mean Ct value of *F. nucleatum*" – "the mean Ct value of *SLCO2A1*"] (8). Each specimen was analyzed in duplicate for each target in a single batch, and the mean of the two Ct values was used for each target. In our previous validation study, the Ct values linearly decreased with the amount of input *F. nucleatum* DNA ($r^2 > 0.99$), and the interassay coefficient of variation of the Ct values in five different batches was <1% for all targets (8). Tumors with any detectable *F. nucleatum* DNA were classified as *F. nucleatum*–positive, whereas all other tumors were classified as *F. nucleatum*–negative.

We performed a quantitative PCR assay to measure the amount of *Bifidobacterium* genus DNA in the tumor using custom TaqMan primer/probe sets (Applied Biosystems) for the 16S ribosomal RNA gene of *Bifidobacterium* genus and for the reference gene 16S (31). The primer and probe sequences for each TaqMan Gene-Expression Assay were as follows: *Bifidobacterium* forward primer, 5′-CGGGTGAGTAATGCGTGACC-3′; *Bifidobacterium* reverse primer, 5′-TGATAGGACGCGACCCCA-3′; *Bifidobacterium* FAM probe, 5′-CTCCTGGAAACGGGTG-3′; universal 16S forward primer, 5′-CGGTGAATACGTTCCCGG-3′; universal 16S reverse primer, 5′-TACGGCTACCTTGTTACGACTT-3′; and universal 16S FAM probe, 5′-CTTGTACACACCGCCCGTC-3′. We used 80 ng DNA in each reaction under the same reaction conditions as for *F. nucleatum* (8). Amounts of intratumoral *Bifidobacterium genus* DNA in each specimen were calculated as a relative value normalized to amounts of 16S using the $2^{-\Delta Ct}$ method (8). Each specimen was analyzed in duplicate for each target in a single batch, and the mean of the two Ct values was used for each target.

Tumors with any detectable *Bifidobacterium* genus DNA were classified as bifidobacteria-positive, whereas all other tumors were classified as bifidobacteria-negative.

MSI status and other molecular characteristics

MSI status was analyzed using 10 microsatellite markers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67, and D18S487), as previously described (29). MSI-high was defined as presence of instability in ≥30% of the markers and non–MSI-high as instability in <30% of the markers. Using bisulfite-treated DNA, we determined methylation status of eight CIMP-specific promoters (*CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, and *SOCS1*) and long interspersed nucleotide element-1 (LINE-1) as previously described (29). CIMP-high was defined as ≥6 methylated promoters of eight promoters, and CIMP-low/negative as 0–5 methylated promoters. PCR and pyrosequencing were performed for *KRAS* (codons 12, 13, 61, and 146), *BRAF* (codon 600), and *PIK3CA* (exons 9 and 20), as previously described (29). We constructed tissue microarrays of colorectal cancer cases with sufficient tissue materials, including up to four tumor cores from each case in one tissue microarray block (32). As previously described (33, 34), IHC for CD274 (PD-L1) and PDCD1LG2 (PDCD1 ligand 2, PD-L2) was performed using an anti-CD274 (clone MIH1; dilution, 1:50; eBioscience) and anti-PDCD1LG2 (clone 366C.9E5; dilution, 1:6,000; provided by the laboratory of G.J. Freeman), respectively. We assessed overall tumor CD274 expression as an ordinal scale of 0 to 4 by summing cytoplasmic expression score [absent (0), weak (1), moderate (2), or strong (3)] and membrane expression score [absent (0) or present (1)], and categorized CD274 as low (overall score of 0 to 1) or high (2 to 4; ref. 33). We calculated the percentage of tumor cells expressing PDCD1LG2 in the cytoplasm or membrane and grouped the data into bins of 0%–20%, 21%–50%, 51%–80%, or 81%–100% (34).

Lymphocytic reaction to colorectal cancer

Lymphocytic reaction to tumor was histopathologically evaluated on whole tissue sections, as previously described (23). Four components of lymphocytic reaction were examined, including tumor-infiltrating lymphocytes (TIL), intratumoral periglandular reaction, peritumoral lymphocytic reaction, and Crohn-like lymphoid reaction (Fig. 1). TIL was defined as lymphocytes on top of cancer cells in a tissue section. Intratumoral periglandular reaction was defined as lymphocytic reaction in tumor stroma within a tumor mass. Peritumoral lymphocytic reaction was defined as discrete lymphoid reaction surrounding a tumor mass. Crohn-like lymphoid reaction was defined as transmural lymphoid reaction. Each of the four components was graded as negative/low, intermediate, or high. A subset of cases ($n = 398$) were independently reviewed by a second pathologist (J.N. Glickman) with a good interobserver correlation as previously described (23). We constructed tissue microarrays and measured densities (cells/mm^2) of CD3$^+$ cells, CD8$^+$ cells, CD45RO (an isoform of the PTPRC protein)$^+$ cells, and FOXP3$^+$ cells in colorectal cancer tissue based on IHC and image analysis using an automated scanning microscope and the Ariol image analysis system (Genetix) as previously described (24).

Statistical analysis

Our primary hypothesis test assessed a statistical interaction between *F. nucleatum* status in colorectal cancer tissue (positive vs. negative) and MSI status (high vs. non-high) in relation to immune response status. We assessed statistical interactions using the Wald test for the cross-product in multivariable-adjusted logistic regression models. In our primary hypothesis testing on new discoveries, we used the α level of 0.005. We conducted all other assessments including evaluations of individual odds ratios (ORs) as secondary analyses, and interpreted the results cautiously in addition to use of the α level of 0.005. Multivariable logistic regression models initially included age (continuous), sex, year of

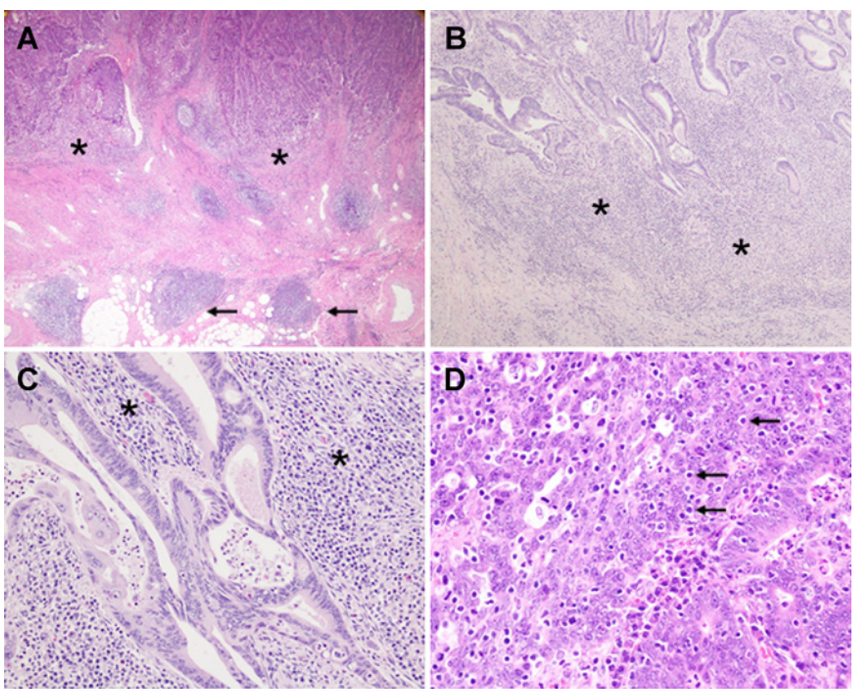

Figure 1.
Lymphocytic reaction to colorectal cancer. **A,** Crohn-like lymphoid reaction (arrows) and peritumoral lymphocytic reaction (asterisks; original magnification, 20×). **B,** Peritumoral lymphocytic reaction (asterisks; original magnification, 40×). **C,** Intratumoral periglandular reaction (asterisks; original magnification, 200×). **D,** Tumor-infiltrating lymphocytes (arrows; original magnification, 400×).

diagnosis (continuous), family history of colorectal cancer (absent vs. present), tumor location (proximal colon vs. distal colon vs. rectum), CIMP status (high vs. low/negative), LINE-1 methylation level (continuous), *KRAS* (mutant vs. wild-type), *BRAF* (mutant vs. wild-type), and *PIK3CA* (mutant vs. wild-type). A backward elimination with a threshold *P* of 0.05 was used to select variables for the final models. Cases with missing data [family history of colorectal cancer (0.8%), tumor location (0.3%), CIMP status (5.4%), *KRAS* (7.7%), *BRAF* (1.1%), and *PIK3CA* (6.6%)] were included in the majority category of a given categorical covariate to limit the degrees of freedom of the models. For cases with missing data on LINE-1 methylation level (2.6%), we assigned a separate indicator variable. We confirmed that the exclusion of cases with missing data in any of the variables did not substantially alter our results. The proportional odds assumption was assessed using the ordinal logistic regression model. We observed evidence of violation of this assumption in three of the four lymphocytic reaction patterns (TIL, intratumoral periglandular reaction, and peritumoral lymphocytic reaction) and T-cell quartile variables. We therefore used binary histologic lymphocytic reaction variables and T-cell variables dichotomized at the median value as outcome variables in logistic regression analyses.

In secondary analyses, we assessed a statistical interaction between *F. nucleatum* status in colorectal cancer tissue (positive vs. negative) and MSI status (high vs. non-high) in relation to colorectal cancer–specific or overall mortality. We assessed statistical interactions using the Wald test for the cross-product in multivariable-adjusted Cox proportional hazards regression models. Multivariable Cox regression models initially included tumor differentiation (well-moderate vs. poor) and disease stage (I/II vs. III/IV) in addition to the same set of covariates as in the multivariable logistic regression models. A backward elimination with a threshold *P* of 0.05 was used to select variables for the final models. Cases with missing data on tumor differentiation (0.1%) were included in the majority category, and the other covariates were dealt as in the multivariable logistic regression models. We confirmed that the exclusion of cases with missing data in any of the variables did not substantially alter our results. The assumption of proportionality of hazards was generally satisfied using the assessment of a time-varying covariate, that is, the cross-product of *F. nucleatum* status and survival time in strata of MSI status ($P > 0.2$). Cumulative survival probabilities were estimated using the Kaplan–Meier method and compared using the log-rank test. For colorectal cancer–specific mortality, deaths from other causes were treated as censored events. Using a single regression model with reparameterization of an interaction term (32), we calculated ORs for immune response status and hazard ratios for colorectal cancer mortality comparing *F. nucleatum*–positive to *F. nucleatum*–negative tumors in the two strata of MSI status.

All statistical analyses were performed using SAS software (version 9.4; SAS Institute), and all *P* values were two-sided.

Results

Among the 1,041 colorectal carcinoma cases from the two prospective cohort studies, *F. nucleatum* DNA was detected using the quantitative PCR assay in 135 (13%) cases. Table 1 shows clinical, pathologic, and molecular features of colorectal cancer cases according to combined tumor tissue *F. nucleatum* and MSI status. As previously reported (8), MSI-high tumors were associated with detectable *F. nucleatum* DNA. There was a trend toward a higher rate of detectable intratumor *F. nucleatum* in more recent cases, particularly for MSI-high tumors. High persistence of *F. nucleatum* has been observed in metastatic liver lesions from *F. nucleatum*–positive colorectal cancer (10). We examined the correlations between *F. nucleatum* and disease stage in strata of tumor MSI status. We observed a significant positive correlation between *F. nucleatum* and disease stage in non–MSI-high tumors ($P = 0.004$, with the α level of 0.005), but not in MSI-high tumors ($P = 0.34$). However, statistical power was limited in the analysis of MSI-high tumors.

Table 2 shows the distribution of colorectal carcinoma cases according to *F. nucleatum* status and histologic lymphocytic reaction patterns in strata of MSI status. The presence of *F. nucleatum* in tumor tissue was negatively correlated with intratumoral periglandular reaction in MSI-high tumors ($P = 0.002$, with the α level of 0.005) and was positively correlated with Crohn-like lymphoid reaction in non–MSI-high tumors ($P = 0.001$).

In our primary hypothesis test using logistic regression models (Table 3; Supplementary Table S1), we observed a statistically significant interaction between *F. nucleatum* and MSI status in relation to TIL or intratumoral periglandular reaction ($P_{\text{interaction}} = 0.002$, with the α level of 0.005). The presence of *F. nucleatum* in tumor tissue was negatively associated with degrees of TIL in MSI-high colorectal cancer [multivariable OR, 0.45; 95% confidence interval (CI), 0.22–0.92], but positively associated with TIL in non–MSI-high cancer (multivariable OR, 1.91; 95% CI, 1.12–3.25). Similarly, the presence of *F. nucleatum* in tumor tissue was negatively associated with intratumoral periglandular reaction in MSI-high tumors (multivariable OR, 0.43; 95% CI, 0.21–0.87), but positively associated with intratumoral periglandular reaction (multivariable OR, 1.97, 95% CI, 1.00–3.86) in non–MSI-high tumors. The statistical interaction of *F. nucleatum* and MSI status in relation to peritumoral lymphocytic reaction or Crohn-like lymphoid reaction was not significant with the α level of 0.005 ($P_{\text{interaction}} > 0.01$).

As secondary analyses using a subset of cases with available tissue microarray data (up to 580 cases), we examined the association of *F. nucleatum* with densities of T-cell subsets ($CD3^+$ cells, $CD8^+$ cells, $CD45RO^+$ cells, or $FOXP3^+$ cells) in strata of MSI status. Although we did not observe a significant interaction between *F. nucleatum* and MSI status in relation to the density of $CD3^+$ cells, $CD8^+$ cells, $CD45RO^+$ cells, or $FOXP3^+$ cells ($P_{\text{interaction}} > 0.1$, with the α level of 0.005; Supplementary Table S2), statistical power was limited in these analyses.

We did not observe a statistically significant interaction between *Bifidobacterium* genus and MSI status in relation to lymphocytic reaction patterns or densities of T-cell subsets ($P_{\text{interaction}} > 0.1$, with the α level of 0.005).

As secondary analyses to assess the prognostic association of *F. nucleatum* in strata of MSI status, we conducted Kaplan–Meier analyses and Cox regression analyses, and did not observe a significant interaction between *F. nucleatum* and MSI status in relation to colorectal cancer–specific or overall mortality (Supplementary Fig. S1; Supplementary Table S3).

Discussion

Using two large prospective cohort studies in the United States, we tested the hypothesis that the association of intratumoral *F. nucleatum* status with local immune response to colorectal cancer might differ by tumor MSI status. We found negative

Table 1. Clinical, pathologic, and molecular characteristics of patients with colorectal cancer and their samples, according to *F. nucleatum* status in tumor tissue, stratified by MSI status

		Non–MSI-high		MSI-high	
		F. nucleatum in tumor		*F. nucleatum* in tumor	
Characteristic	**All cases[a] (*n* = 1,041)**	**Negative[a] (*n* = 787)**	**Positive[a] (*n* = 77)**	**Negative[a] (*n* = 119)**	**Positive[a] (*n* = 58)**
Sex					
Female (NHS)	609 (59%)	437 (56%)	42 (55%)	85 (71%)	45 (78%)
Male (HPFS)	432 (41%)	350 (44%)	35 (45%)	34 (29%)	13 (22%)
Mean age ± SD (years)	69.5 ± 8.9	68.9 ± 9.0	69.5 ± 9.6	72.1 ± 7.6	72.5 ± 8.4
Race/ethnicity					
White	995 (97%)	751 (97%)	74 (99%)	114 (97%)	56 (98%)
Black	16 (1.6%)	13 (1.7%)	0	2 (1.7%)	1 (1.8%)
Other	11 (1.1%)	8 (1.0%)	1 (1.3%)	2 (1.7%)	0
Year of diagnosis					
1995 or before	343 (33%)	285 (36%)	21 (27%)	29 (24%)	8 (14%)
1996–2000	286 (27%)	216 (27%)	25 (32%)	33 (28%)	12 (21%)
2001–2012	412 (40%)	286 (36%)	31 (40%)	57 (48%)	38 (66%)
Family history of colorectal cancer					
In first-degree relative(s)					
Absent	829 (80%)	632 (81%)	66 (88%)	84 (71%)	47 (81%)
Present	204 (20%)	150 (19%)	9 (12%)	34 (29%)	11 (19%)
Prediagnosis body mass index[b]					
<25 kg/m^2	417 (40%)	311 (40%)	30 (39%)	49 (42%)	27 (47%)
25–29.9 kg/m^2	424 (41%)	330 (42%)	35 (46%)	43 (36%)	16 (28%)
≥30 kg/m^2	194 (19%)	142 (18%)	11 (14%)	26 (22%)	15 (26%)
Prudent dietary pattern[c]					
Quartile 1 (lowest)	258 (25%)	187 (24%)	25 (33%)	30 (25%)	16 (28%)
Quartile 2	258 (25%)	206 (26%)	19 (25%)	21 (18%)	12 (21%)
Quartile 3	257 (25%)	187 (24%)	21 (28%)	32 (27%)	17 (29%)
Quartile 4 (highest)	258 (25%)	199 (26%)	11 (14%)	35 (30%)	13 (22%)
Western dietary pattern[c]					
Quartile 1 (lowest)	258 (25%)	198 (25%)	10 (13%)	31 (26%)	19 (33%)
Quartile 2	258 (25%)	192 (25%)	21 (28%)	32 (27%)	13 (22%)
Quartile 3	258 (25%)	200 (26%)	20 (26%)	21 (18%)	17 (29%)
Quartile 4 (highest)	258 (25%)	190 (24%)	25 (33%)	34 (29%)	9 (16%)
Tumor location					
Proximal colon	522 (50%)	332 (42%)	33 (43%)	104 (87%)	53 (91%)
Distal colon	297 (29%)	264 (34%)	20 (26%)	10 (8.4%)	3 (5.2%)
Rectum	219 (21%)	189 (24%)	23 (30%)	5 (4.2%)	2 (3.5%)
Tumor differentiation					
Well to moderate	936 (90%)	745 (95%)	67 (88%)	86 (72%)	38 (66%)
Poor	104 (10%)	42 (5.3%)	9 (12%)	33 (28%)	20 (34%)
AJCC disease stage					
I	239 (25%)	197 (27%)	8 (11%)	25 (22%)	9 (17%)
II	314 (33%)	199 (28%)	21 (30%)	64 (56%)	30 (56%)
III	280 (29%)	222 (31%)	28 (39%)	18 (16%)	12 (22%)
IV	127 (13%)	103 (14%)	14 (20%)	7 (6.1%)	3 (5.6%)
CIMP status					
CIMP-low/negative	794 (81%)	688 (92%)	68 (94%)	24 (21%)	14 (25%)
CIMP-high	191 (19%)	56 (7.5%)	4 (5.6%)	89 (79%)	42 (75%)
Mean LINE-1 methylation level ± SD (%)	63.6 ± 10.4	62.6 ± 10.2	62.2 ± 9.4	68.4 ± 10.3	70.5 ± 9.5
KRAS mutation					
Wild-type	541 (56%)	390 (52%)	30 (41%)	88 (85%)	33 (82%)
Mutant	420 (44%)	353 (48%)	44 (59%)	16 (15%)	7 (18%)
BRAF mutation					
Wild-type	858 (83%)	709 (91%)	73 (95%)	55 (47%)	21 (37%)
Mutant	172 (17%)	70 (9.0%)	4 (5.2%)	62 (53%)	36 (63%)
PIK3CA mutation					
Wild-type	812 (84%)	617 (84%)	59 (81%)	91 (84%)	45 (85%)
Mutant	160 (16%)	121 (16%)	14 (19%)	17 (16%)	8 (15%)
Tumor CD274 (PD-L1) expression					
Low	263 (41%)	176 (37%)	29 (52%)	37 (46%)	21 (64%)
High	377 (59%)	294 (63%)	27 (48%)	44 (54%)	12 (36%)
Tumor PDCD1LG2 (PD-L2) expression					
0%–20%	160 (25%)	111 (24%)	19 (37%)	22 (28%)	8 (25%)
21%–50%	148 (23%)	110 (23%)	11 (21%)	17 (21%)	10 (31%)
51%–80%	197 (31%)	150 (32%)	12 (23%)	25 (31%)	10 (31%)
81%–100%	129 (20%)	99 (21%)	10 (19%)	16 (20%)	4 (13%)
Bifidobacterium genus DNA					
Negative	748 (73%)	576 (75%)	54 (71%)	82 (69%)	36 (63%)
Positive	276 (27%)	197 (25%)	22 (29%)	36 (31%)	21 (37%)

Abbreviations: AJCC, American Joint Committee on Cancer; CIMP, CpG island methylator phenotype; LINE-1, long interspersed nucleotide element-1; SD, standard deviation.

[a]Percentage indicates the proportion of cases with a specific clinical, pathologic, or molecular characteristic in all cases or in strata of *F. nucleatum* and MSI status. Total percentages may not equal 100% due to rounding.

[b]Calculated by dividing weight (kilogram) by squared height (meter).

[c]The prudent dietary pattern is characterized by high intake of vegetables, fruits, fish, poultry, and whole grains, and the Western dietary pattern is characterized by high intake of red and processed meats, added sugar, and refined grains (42).

Table 2. Distribution of colorectal cancer samples according to *F. nucleatum* status in tumor tissue and lymphocytic reaction status, stratified by MSI status

			F. nucleatum in colorectal cancer tissue		
		All cases[a]	Negative[a]	Positive[a]	*P*[b]
Non–MSI-high	TILs ($n = 862$)				0.013
	Negative/low	699 (81%)	644 (82%)	55 (71%)	
	Intermediate	119 (14%)	107 (14%)	12 (16%)	
	High	44 (5.1%)	34 (4.3%)	10 (13%)	
	Intratumoral periglandular reaction ($n = 863$)				0.57
	Negative/low	124 (14%)	106 (13%)	18 (23%)	
	Intermediate	660 (76%)	613 (78%)	47 (61%)	
	High	79 (9.2%)	67 (8.5%)	12 (16%)	
	Peritumoral lymphocytic reaction ($n = 859$)				0.89
	Negative/low	138 (16%)	123 (16%)	15 (20%)	
	Intermediate	615 (72%)	567 (72%)	48 (63%)	
	High	106 (12%)	93 (12%)	13 (17%)	
	Crohn-like lymphoid reaction ($n = 723$)				0.001
	Negative/low	586 (81%)	544 (83%)	42 (66%)	
	Intermediate	111 (15%)	92 (14%)	19 (30%)	
	High	26 (3.6%)	23 (3.5%)	3 (4.7%)	
MSI-high	TILs ($n = 177$)				0.055
	Negative/low	46 (26%)	25 (21%)	21 (36%)	
	Intermediate	64 (36%)	45 (38%)	19 (33%)	
	High	67 (38%)	49 (41%)	18 (31%)	
	Intratumoral periglandular reaction ($n = 177$)				0.002
	Negative/low	12 (6.8%)	1 (0.8%)	11 (19%)	
	Intermediate	100 (56%)	68 (57%)	32 (55%)	
	High	65 (37%)	50 (42%)	15 (26%)	
	Peritumoral lymphocytic reaction ($n = 177$)				0.062
	Negative/low	13 (7.3%)	4 (3.4%)	9 (16%)	
	Intermediate	91 (51%)	62 (52%)	29 (50%)	
	High	73 (41%)	53 (45%)	20 (34%)	
	Crohn-like lymphoid reaction ($n = 151$)				0.13
	Negative/low	64 (42%)	41 (41%)	23 (44%)	
	Intermediate	48 (32%)	26 (26%)	22 (42%)	
	High	39 (26%)	32 (32%)	7 (13%)	

[a]Percentage indicates the proportion of cases with a specific level of lymphocytic reaction in strata of MSI status or in strata of *F. nucleatum* and MSI status. Total percentages may not equal 100% due to rounding.

[b]*P* value was calculated using the Spearman correlation test between *F. nucleatum* status (positive vs. negative) and each lymphocytic reaction variable [ordinal; negative/low (0), intermediate (1), and high (2)].

associations of *F. nucleatum* status with degrees of TIL and intratumoral periglandular reaction in MSI-high colorectal carcinomas and positive associations with those reaction patterns in non–MSI-high carcinomas. Our findings suggest an interplay between *F. nucleatum*, MSI status, and immune cells in colorectal tumor.

High-level infiltration of lymphocytes in colorectal cancer has been associated with better clinical outcomes (23, 24, 35, 36), and therapeutic activation of antitumor immune response in the tumor microenvironment has become an attractive strategy for cancer immunotherapy (37–39). In particular, immune-checkpoint inhibitors that target the PDCD1 (PD-1) or CD274 (PDCD1 ligand 1, PD-L1) protein have shown promise in treating various cancer types (37–39), including colorectal cancer with high MSI (26–28). However, despite the accompanying intense immune response, some MSI-high colorectal cancers can evade the immune eradication, and not all MSI-high tumors respond to the immune-checkpoint blockade. Therefore, immunosuppressive factors in MSI-high colorectal cancer need to be elucidated to improve the efficacy of immunotherapies in colorectal cancer. In the tumor microenvironment, host cells, neoplastic cells, and microbiota form complex interactive networks. A better understanding of these interactions would have relevance to existing avenues of treatment and may also reveal additional strategies for combating cancer (40, 41).

Alterations in the gut microbial composition are considered to play a role in colorectal cancer development (3, 42, 43). Fiber-rich diets are associated with a lower risk of colorectal cancer containing abundant *F. nucleatum* (42). Our findings of a possible temporal increase in detectable intratumor *F. nucleatum* might be due to growing popularity of colonoscopy screening, changes in dietary and lifestyle patterns, and/or aging of the cohort participants. Such interactions might become evident with a longitudinal epidemiologic investigation. Mechanistic studies indicate that *F. nucleatum* can exert carcinogenic effects through binding of its adhesin FadA to CDH1 (E-cadherin) and resultant activation of the CTNNB1 (beta-catenin)/WNT signaling pathway in colorectal carcinoma cells (44) as well as through suppression of the adaptive immune response (12). WNT signaling activation has been linked to immunosuppression in colorectal carcinoma (45). Experimental evidence suggests that binding of the virulence factor FAP2 to a host factor Gal-GalNAc may lead to interaction with the inhibitory immune cell receptor TIGIT, thereby inhibiting T-cell activity and NK cell cytotoxicity (11, 13, 46). In line with these studies, our previous population-based study has shown inverse associations of the enrichment of *F. nucleatum* in colorectal cancer tissue with $CD3^+$ pan-T-cell density and patient survival (8, 18). *F. nucleatum* also increases numbers of myeloid-derived suppressor cells and tumor-associated macrophages in the colorectal tumor microenvironment (12, 47), contributing to

Table 3. Logistic regression analyses to assess the association of *F. nucleatum* status in colorectal cancer tissue with lymphocytic reaction status, in strata of MSI status

		Univariable OR (95% CI)	Multivariable OR (95% CI)[a]
Model for TILs[b] (as an outcome variable; $n = 1{,}039$)			
Non–MSI-high	*F. nucleatum*-negative	1 (referent)	1 (referent)
	F. nucleatum-positive	1.83 (1.08–3.09)	1.91 (1.12–3.25)
MSI-high	*F. nucleatum*-negative	1 (referent)	1 (referent)
	F. nucleatum-positive	0.47 (0.23–0.94)	0.45 (0.22–0.92)
$P_{interaction}$[c]		0.002	0.002
Model for intratumoral periglandular reaction[b] (as an outcome variable; $n = 1{,}040$)			
Non–MSI-high	*F. nucleatum*-negative	1 (referent)	1 (referent)
	F. nucleatum-positive	1.98 (1.02–3.85)	1.97 (1.00–3.86)
MSI-high	*F. nucleatum*-negative	1 (referent)	1 (referent)
	F. nucleatum-positive	0.48 (0.24–0.96)	0.43 (0.21–0.87)
$P_{interaction}$[c]		0.004	0.002
Model for peritumoral lymphocytic reaction[b] (as an outcome variable; $n = 1{,}036$)			
Non–MSI-high	*F. nucleatum*-negative	1 (referent)	1 (referent)
	F. nucleatum-positive	1.53 (0.81–2.89)	1.44 (0.76–2.76)
MSI-high	*F. nucleatum*-negative	1 (referent)	1 (referent)
	F. nucleatum-positive	0.66 (0.34–1.26)	0.54 (0.27–1.05)
$P_{interaction}$[c]		0.068	0.038
Model for Crohn-like lymphoid reaction[b] (as an outcome variable; $n = 874$)			
Non–MSI-high	*F. nucleatum*-negative	1 (referent)	1 (referent)
	F. nucleatum-positive	2.48 (1.42–4.31)	2.86 (1.62–5.06)
MSI-high	*F. nucleatum*-negative	1 (referent)	1 (referent)
	F. nucleatum-positive	0.89 (0.45–1.76)	0.99 (0.49–1.98)
$P_{interaction}$[c]		0.022	0.020

[a]The multivariable logistic regression model initially included sex, age at diagnosis, year of diagnosis, family history of colorectal cancer, tumor location, CpG island methylator phenotype-specific promoter status, long interspersed nucleotide element-1 methylation level, and *KRAS, BRAF*, and *PIK3CA* mutations. A backward elimination with a threshold *P* of 0.05 was used to select variables for the final models. The variables that remained in the final models for TILs and intratumoral periglandular reaction were shown in Supplementary Table S1.

[b]To avoid violation of the proportional odds assumption, the most common score or less (i.e., 0 for TILs and Crohn-like lymphoid reaction; and 0–1 for intratumoral periglandular reaction and peritumoral lymphocytic reaction) was categorized as low, and higher score was categorized as high.

[c]$P_{interaction}$ (two-sided) was calculated using the Wald test for the cross-product of *F. nucleatum* status (positive vs. negative) and MSI status (high vs. non-high) in the logistic regression model.

proinflammatory reactions and impairment of tumor-specific immune responses.

Previous studies have linked *F. nucleatum* to MSI-high colorectal cancer (8, 16, 17), which is characterized by a vigorous antitumor immune response in the tumor microenvironment resulting from frameshift mutations and high expression of immunogenic peptides (23–27). However, there is a paradoxical finding that the presence of detectable *F. nucleatum* in tumor tissue is inversely associated with $CD3^+$ T-cell density (8). Our current findings suggest that *F. nucleatum* may exert suppressive effects on adaptive antitumor immune response in MSI-high colorectal cancer with abundant immunogenic neoantigens (Fig. 2). During the development of colorectal carcinomas, MSI-high tumors need to acquire the means to resist antitumor immune activity. Our study provides evidence suggesting that infection with *F. nucleatum* may promote immune evasion in the immune cell–rich microenvironment of MSI-high colorectal carcinomas. We did not observe differential associations of *F. nucleatum* with T-cell subsets ($CD3^+$ cells, $CD8^+$ cells, $CD45RO^+$ cells, and $FOXP3^+$ cells) by tumor MSI status. These seemingly conflicting findings might be due to the roles of other cell types in immune reactions to tumor, measurement errors in T-cell densities, and/or a smaller overall sample size in analyses of T cells.

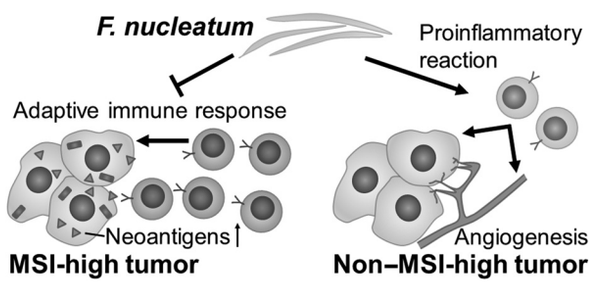

Figure 2.

F. nucleatum and lymphocytic reaction to colorectal carcinoma according to tumor MSI status. The current study suggests that *F. nucleatum* may attenuate adaptive immune response to MSI-high colorectal carcinomas, which exhibit abundant immunogenic neoantigens. In contrast, the proinflammatory properties of *F. nucleatum* may lead to lymphocytic reaction to non–MSI-high carcinomas that contain lower amounts of neoantigens and tumor-infiltrating lymphocytes than MSI-high carcinomas.

The trend toward more lymphocytic reaction associated with *F. nucleatum* in non–MSI-high tumors warrants further discussion. In addition to suppression of the antitumor immune response, *F. nucleatum* can exert proinflammatory effects in the colorectal mucosa (12, 14, 15) and has been implicated in pathogenesis of inflammatory bowel diseases (14). Studies have shown that *F. nucleatum* may increase expression of proinflammatory cytokines and upregulate the NF-κB pathway (12, 48) in colorectal tumors. Therefore, we speculate that the proinflammatory effects of *F. nucleatum* may outweigh its immunosuppressive effects in non–MSI-high tumors, which contain fewer neoantigens and

fewer tumor-infiltrating immune cells on average relative to MSI-high tumors (Fig. 2). These divergent associations of tumor tissue *F. nucleatum* with lymphocytic reactions between MSI-high and non–MSI-high tumors underscore the importance of interactive effects of environmental and tumor molecular features on host immune response in the tumor microenvironment (40).

We acknowledge limitations of our study. Our study focused on limited numbers of microbial species and immune reaction components. Accumulating evidence points to the roles of a variety of microbes and immune cells in antitumor immune responses and carcinogenesis (2, 3, 49). Therefore, more comprehensive analyses are warranted to investigate the interactions between microbes other than *F. nucleatum* (e.g., enterotoxigenic *Bacteroides fragilis* and pks-positive *Escherichia coli*; ref. 4) and other immune parameters (e.g., myeloid-derived suppressor cells, tumor-associated macrophages, and TIGIT-expressing cells). High-throughput sequencing-based analyses including 16S rRNA gene sequencing, metagenomic analyses, gene-expression profiling, and T-cell receptor sequencing would also provide insights into the interrelationship of tumor molecular markers and microbiota in relation to antitumor immune response. Detailed data on antibiotics and cancer treatments including immune-checkpoint inhibitors were not available in our study. There might be confounding factors that were unaccounted for; nonetheless, we adjusted for a variety of demographic, clinical, and tumor characteristics, as we utilized the MPE database. Another limitation is the cross-sectional study design, which dictates that we cannot rule out reverse causation. It is possible that immune responses may alter the amount of *F. nucleatum* in the tumor microenvironment. However, our hypothesis was based on accumulating evidence for the suppressive effects of *F. nucleatum* on T-cell–mediated antitumor immunity (8, 11–13). We used the quantitative PCR assay for *F. nucleatum* in FFPE tissue specimens; therefore, tissue processing procedures and storage conditions might have affected the detection rate. Nonetheless, our previous validation study using quantitative PCR showed concordance in detecting *F. nucleatum* in paired FFPE and frozen tissue specimens and linearity and reproducibility of *F. nucleatum* measurements in FFPE tissue specimens (8). Finally, our findings need to be validated in independent cohorts.

The current study has strengths, including the use of the MPE (40, 41) database derived from two U.S. prospective cohort studies, and this study represents the integration of immunology into the MPE paradigm (i.e., immunology-MPE; ref. 50). The data on the amount of *F. nucleatum* DNA in the tumor, tumor molecular characteristics, and pathologic findings in over 1,000 cases allowed us to comprehensively examine possible differential roles of *F. nucleatum* in MSI-high and non–MSI-high colorectal cancers. Our study population was derived from a large number of incident colorectal cancer cases that occurred in the well-defined populations of the prospective cohorts, and cases were also derived from hospitals in 48 states in the United States; both features increase the generalizability of our findings. Nevertheless, our findings need to be validated in independent studies.

In conclusion, the current study provides evidence for divergent effects of *F. nucleatum* in the tumor microenvironment according to tumor MSI status. There may be dominant suppressive effects of *F. nucleatum* on adaptive antitumor immune responses in MSI-high colorectal cancer and apparent proinflammatory effects in non–MSI-high cancer. Our findings, if validated, would inform future mechanistic studies examining the interplay of *F. nucleatum* and tumor characteristics in colorectal tumor evolution.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

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Authors' Contributions

Conception and design: T. Hamada, X. Zhang, K. Mima, K. Kosumi, M. Song, L. Liu, A.D. Kostic, W.S. Garrett, M. Meyerson, E.L. Giovannucci, M. Giannakis, S. Ogino

Development of methodology: T. Hamada, K. Mima, S. Bullman, Y. Sukawa, K. Kosumi, L. Liu, C. Huttenhower, W.S. Garrett, A.T. Chan, S. Ogino

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Hamada, K. Mima, Y. Sukawa, K. Kosumi, Y. Masugi, Y. Shi, M. Gu, W. Li, H. Koh, K. Inamura, E.L. Giovannucci, A.T. Chan, C.S. Fuchs, M. Giannakis, S. Ogino

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Zhang, K. Mima, S. Bullman, J.A. Nowak, K. Kosumi, Y. Cao, M. Song, L. Liu, Y. Shi, H. Koh, K. Nosho, N. Keum, K. Wu, J.A. Meyerhardt, C.S. Fuchs, R. Nishihara, M. Giannakis, S. Ogino

Writing, review, and/or revision of the manuscript: T. Hamada, X. Zhang, S. Bullman, J.A. Nowak, K. Kosumi, Y. Masugi, T.S. Twombly, Y. Cao, M. Song, L. Liu, A. da Silva, Y. Shi, M. Gu, H. Koh, N. Keum, K. Wu, J.A. Meyerhardt, W.S. Garrett, M. Meyerson, E.L. Giovannucci, A.T. Chan, M. Giannakis, S. Ogino

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Sukawa, Y. Masugi, L. Liu, A. da Silva, A.T. Chan, S. Ogino

Study supervision: J.A. Nowak, A.T. Chan, C.S. Fuchs, M. Giannakis, S. Ogino

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