ORIGINAL ARTICLE

Fusobacterium nucleatum in colorectal carcinoma tissue and patient prognosis

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ABSTRACT

Objective Accumulating evidence links the intestinal microbiota and colorectal carcinogenesis. *Fusobacterium nucleatum* may promote colorectal tumour growth and inhibit T cell-mediated immune responses against colorectal tumours. Thus, we hypothesised that the amount of *F. nucleatum* in colorectal carcinoma might be associated with worse clinical outcome.

Design We used molecular pathological epidemiology database of 1069 rectal and colon cancer cases in the Nurses' Health Study and the Health Professionals Follow-up Study, and measured *F. nucleatum* DNA in carcinoma tissue. Cox proportional hazards model was used to compute hazard ratio (HR), controlling for potential confounders, including microsatellite instability (MSI, mismatch repair deficiency), CpG island methylator phenotype (CIMP), *KRAS*, *BRAF*, and *PIK3CA* mutations, and LINE-1 hypomethylation (low-level methylation).

Results Compared with *F. nucleatum*-negative cases, multivariable HRs (95% CI) for colorectal cancer-specific mortality in *F. nucleatum*-low cases and *F. nucleatum*-high cases were 1.25 (0.82 to 1.92) and 1.58 (1.04 to 2.39), respectively, (p for trend=0.020). The amount of *F. nucleatum* was associated with MSI-high (multivariable odd ratio (OR), 5.22; 95% CI 2.86 to 9.55) independent of CIMP and *BRAF* mutation status, whereas CIMP and *BRAF* mutation were associated with *F. nucleatum* only in univariate analyses (p<0.001) but not in multivariate analysis that adjusted for MSI status.

Conclusions The amount of *F. nucleatum* DNA in colorectal cancer tissue is associated with shorter survival, and may potentially serve as a prognostic biomarker. Our data may have implications in developing cancer prevention and treatment strategies through targeting GI microflora by diet, probiotics and antibiotics.

Significance of this study

What is already known on this subject?

- Microorganisms play an important role in health and disease conditions, including cancer.
- *Fusobacterium nucleatum* has been shown to promote colorectal tumour growth and inhibit antitumour immune responses in animal models.
- *F. nucleatum* DNA is detectable in a subset of human colorectal neoplasias.

What are the new findings?

- The amount of *F. nucleatum* DNA in colorectal cancer tissue is positively associated with colorectal cancer-specific mortality, independent of clinical, pathological and major tumour molecular features.
- The amount of *F. nucleatum* DNA in colorectal cancer tissue is positively associated with pT stage.
- The amount of *F. nucleatum* in colorectal cancer tissue is associated with microsatellite instability (MSI)-high in univariable and multivariable analyses (independent of CpG island methylator phenotype (CIMP) and *BRAF* mutation status), whereas CIMP and *BRAF* mutation are associated with *F. nucleatum* only in univariate analyses but not after adjusting for MSI status.

How might it impact on clinical practice in the foreseeable future?

- *F. nucleatum* DNA in colorectal carcinoma tissue may serve as a potential prognostic biomarker.
- Our population-based data can provide insights for the development of new colorectal cancer prevention and treatment strategies through targeting the microbiota.

INTRODUCTION

More than 100 trillion (10^{14}) microorganisms inhabit the human intestinal tract and play an important role in health and disease conditions, including cancer.[1–4] A growing body of evidence suggests a potential link between the microbiota and colorectal carcinogenesis.[5–13] Proportions of colorectal cancers with specific molecular features including microsatellite instability (MSI), CpG island methylator phenotype (CIMP)-high and *BRAF* mutation have been shown to decrease

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continuously from ascending colon to rectum, supporting a gradual change in pathogenic influence of intestinal microbiota and luminal contents along the proximal-distal axis.[14]

Studies have demonstrated an enrichment of *Fusobacterium nucleatum* in human colorectal adenomas and carcinomas compared with adjacent normal tissue.[15–17] Experimental studies have shown that *F. nucleatum* activates the WNT signalling pathway in colorectal carcinoma cells and may promote colorectal tumour growth,[18] and that *F. nucleatum* may inhibit T cell-mediated immune responses against colorectal tumours.[16 19] Consistent with these lines of experimental evidence, a higher amount of tissue *F. nucleatum* DNA has been associated with advanced disease stage[6 7 20] and a lower density of T cells in human colorectal carcinoma tissue.[21] However, the prognostic significance of *F. nucleatum* DNA in colorectal cancer tissue, controlling for clinical, pathological and tumour molecular features, remains uncertain. We hypothesised that a higher amount of tissue *F. nucleatum* DNA might be associated with worse clinical outcome in colorectal cancer.

To test this hypothesis, we used over 1000 colorectal carcinoma cases in two US nationwide prospective cohort studies (the Nurses' Health Study and the Health Professionals Follow-up Study), and examined the amount of tissue *F. nucleatum* DNA in relation to colorectal cancer mortality. Use of our comprehensive database enabled us to examine its prognostic role, while controlling for potential confounders including statuses of MSI, CIMP and *BRAF* mutation.

METHODS

Study population

We used the database of two US nationwide prospective cohort studies, the Nurses' Health Study (with 121 701 women enrolled in 1976) and the Health Professionals Follow-up Study (with 51 529 men enrolled in 1986).[22 23] Every 2 years, participants were sent follow-up questionnaires to gather information on health and lifestyle factors, and asked whether they had received diagnoses of major disease including cancers. The National Death Index was used to ascertain deaths of study participants and identify fatal colorectal carcinoma cases. Follow-up has exceeded 90% for each 2-year questionnaire. Study physicians reviewed medical records for all colorectal cancer cases, and assigned the cause of death for all deceased cases. Formalin-fixed paraffin-embedded (FFPE) tissue blocks were collected from hospitals where participants with colorectal carcinoma had undergone tumour resection. We included colon and rectal carcinoma cases, considering the colorectal continuum model.[24] A single pathologist (SO), who was unaware of other data, conducted a centralised review of H&E-stained tissue sections from all colorectal carcinoma cases, and recorded pathological features. Tumour differentiation was categorised as well to moderate or poor (>50% vs ≤50% glandular area). We analysed available data on tissue *F. nucleatum* DNA and patient survival in 1069 colorectal carcinoma cases diagnosed up to 2008. Written informed consent was obtained from all study participants.

Quantitative PCR for *F. nucleatum*

DNA was extracted from colorectal carcinoma tissue in whole-tissue sections of FFPE tissue blocks using QIAamp DNA FFPE Tissue Kit (Qiagen). We performed a quantitative PCR assay to measure the amount of tissue *F. nucleatum* DNA, after assay validation as previously described.[21] Custom TaqMan primer/probe sets (Applied Biosystems) for the *nusG* gene of *F. nucleatum* and for the reference human gene, *SLCO2A1* were used as previously described.[7] Each reaction contained 80 ng of genomic DNA and was assayed in 20 µL reactions containing 1× final concentration TaqMan Environmental Master Mix 2.0 (Applied Biosystems) and each TaqMan Gene Expression Assay (Applied Biosystems), in a 96-well optical PCR plate. Amplification and detection of DNA was performed with the StepOnePlus real-time PCR Systems (Applied Biosystems) using the following reaction conditions: 10 min at 95°C and 45 cycles of 15 s at 95°C and 1 min at 60°C.

Our validation study has previously shown that in colorectal carcinoma cases with detectable *F. nucleatum* DNA, the cycle threshold (Ct) values in the quantitative PCR for *F. nucleatum* and *SLCO2A1* decreased linearly with the log-transformed amount of input DNA from the same specimen (r^2>0.99), and that the interassay coefficient of variation of Ct values from the same specimen in five different batches was 1% or less for all targets.[21] Each specimen was analysed in duplicate for each target in a single batch, and we used the mean of the two Ct values for each target. The amount of tissue *F. nucleatum* DNA in each specimen was calculated as a relative unitless value normalised with *SLCO2A1* using the $2^{-\Delta Ct}$ method (where ΔCt='the mean Ct value of *F. nucleatum*'—'the mean Ct value of *SLCO2A1*').[21]

Analyses of MSI, DNA methylation, and *KRAS*, *BRAF* and *PIK3CA* mutations

DNA was extracted from colorectal carcinoma tissue in whole-tissue sections from FFPE tissue blocks. MSI status was analysed with the use of 10 microsatellite markers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67 and D18S487) as previously described.[25] We defined MSI-high as the presence of instability in ≥30% of the markers, and MSI-low/microsatellite stable (MSS) as instability in <30% of the markers. Methylation analyses of long interspersed nucleotide element-1 (LINE-1)[26 27] and eight promoter CpG islands specific for CIMP (*CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*)[28 29] were performed. PCR reaction and pyrosequencing were performed for *KRAS* (codons 12, 13, 61 and 146),[30 31] *BRAF* (codon 600)[25] and *PIK3CA* (exons 9 and 20).[32 33]

Statistical analysis

All statistical analyses were conducted using SAS (V.9.3, SAS Institute, Cary, North Carolina, USA) and all p values were two-sided. Our primary hypothesis testing was a linear trend test in Cox proportional hazards regression model to assess an association of the amount of tissue *F. nucleatum* DNA with colorectal cancer-specific mortality. Overall mortality was a secondary outcome. Cases with detectable *F. nucleatum* DNA were categorised as low versus high based on the median cut point amount of *F. nucleatum* DNA, while cases without detectable *F. nucleatum* DNA were categorised as negative. Test for a linear trend was conducted across the ordinal categories (negative (0), low (1), and high (2)) of the amount of tissue *F. nucleatum* DNA as a continuous variable in the Cox proportional hazards regression model. A two-sided α level was set at 0.05 for our primary hypothesis testing.

For analyses of colorectal cancer-specific mortality, deaths as a result of other causes were censored. To control for confounders, we used multivariable Cox proportional hazards regression models. Multivariable models included disease stage as a stratifying variable using strata function in the SAS proc phreg command. In addition to the amount of tissue *F. nucleatum* DNA, the multivariable model initially included sex, age at diagnosis (continuous), year of diagnosis (continuous), family

history of colorectal cancer in a first-degree relative (present vs absent), tumour location (proximal colon vs distal colon vs rectum), MSI (high vs low/MSS), CIMP (high vs low/negative), *KRAS* (mutant vs wild type), *BRAF* (mutant vs wild type), *PIK3CA* (mutant vs wild type) and tumour LINE-1 methylation level (continuous). A backward stepwise elimination with a threshold of p=0.05 was used to select variables in the final models. For cases with missing information on LINE-1 methylation level (5.1%), we assigned a separate indicator variable. For cases with missing information in any of the categorical covariates (family history of colorectal cancer in a first-degree relative (0.4%), tumour location (0.3%), MSI (4.3%), CIMP (8.5%), *KRAS* (7.5%), *BRAF* (3.6%) and *PIK3CA* (9.3%)), we included these cases in the majority category of a given covariate to minimise the number of variables in multivariable Cox models. We confirmed that excluding the cases with missing information in any of the covariates did not substantially alter results (data not shown). Previous experimental studies provide evidence for potentiating effects of *F. nucleatum* on colorectal tumour progression.[16 18] If the hypothesis that tissue *F. nucleatum* is associated with shorter survival is true, high disease stage and poor tumour differentiation (both of which are associated with tissue *F. nucleatum* in the current study) are likely mediators on the causal pathway from the amount of tissue *F. nucleatum* DNA to shorter survival. Thus, we did not include disease stage or tumour differentiation in multivariable Cox proportional hazards regression models in our secondary analysis. The proportionality of hazards assumption was assessed by a time-varying covariate, using an interaction term of colorectal cancer-specific survival term and the amount of *F. nucleatum* DNA (p=0.45). The Kaplan-Meier method was used to describe the distribution of colorectal cancer-specific survival and overall survival, and the log-rank test for trend was performed to assess a linear trend in survival probability across the ordinal categories (negative (0), low (1) and high (2)) of the relative amount of tissue *F. nucleatum* DNA.

All cross-sectional univariable analyses for clinical, pathological and tumour molecular associations were secondary exploratory analyses, with an adjusted two-sided α level of 0.003 (=0.05/16) for multiple hypothesis testing. To assess associations between the ordinal categories of the amount of tissue *F. nucleatum* DNA and other categorical variables, Fisher's exact test was performed. To compare mean age and mean LINE-1 methylation levels, an analysis of variance assuming equal variances was performed.

We conducted logistic regression analyses to assess associations of the amount of tissue *F. nucleatum* DNA (an ordinal predictor variable (negative, low and high)) with each component of the American Joint Committee on Cancer staging system, including pT stage (an ordinal outcome variable (pT1 vs pT2 vs pT3 vs pT4)), pN stage (an ordinal outcome variable (pN0 vs pN1 vs pN2)), and M stage (a binary outcome variable (M0 vs M1)). Test for a linear trend was conducted across the ordinal categories (negative (0), low (1) and high (2)) of the amount of tissue *F. nucleatum* DNA as a continuous variable in logistic regression models. The multivariable logistic regression model initially included age (continuous), sex, year of diagnosis (continuous), family history of colorectal carcinoma in a first-degree relative (present vs absent), tumour location (proximal colon vs distal colon vs rectum), MSI (high vs low/MSS), CIMP (high vs low/negative), *KRAS* (mutant vs wild type), *BRAF* (mutant vs wild type), *PIK3CA* (mutant vs wild type) and LINE-1 methylation level (continuous). For cases with missing information in any of the covariates, we assigned a separate ('missing') indicator variable. A backward stepwise elimination with a threshold of p=0.05 was used to select variables in the final models. To assess independent associations of MSI, CIMP and *BRAF* mutation status (predictor variables) with the amount of tissue *F. nucleatum* DNA (an ordinal outcome variable (negative vs low vs high)), we performed multivariable ordinal logistic regression analysis. In addition to MSI, CIMP and *BRAF* mutation status, the multivariable ordinal logistic regression model initially included age (continuous), sex, year of diagnosis (continuous), family history of colorectal carcinoma in a first-degree relative (present vs absent), tumour location (proximal colon vs distal colon vs rectum), *KRAS* (mutant vs wild type), *PIK3CA* (mutant vs wild type) and LINE-1 methylation level (continuous). For cases with missing information in any of the covariates, we assigned a separate ('missing') indicator variable. A backward stepwise elimination was performed with a threshold of p=0.05 to select covariates in the final model. We assessed the proportional odds assumption in the ordinal logistic regression model, which was generally satisfied (p≥0.06).

RESULTS

F. nucleatum in colorectal cancer tissue and patient mortality

We measured the relative amount of *F. nucleatum* DNA in tumour tissue of 1069 colorectal carcinoma cases within the Nurses' Health Study and the Health Professionals Follow-up Study, using the quantitative PCR assay as previously described.[21] Table 1 shows clinical, pathological and tumour molecular features of the 1069 cases.

F. nucleatum DNA was detected in colorectal carcinoma tissue in 134 (13%) of the 1069 cases. We equally dichotomised the cases with detectable *F. nucleatum* DNA into low versus high.

To test our primary hypothesis, we examined the relationship between the relative amount of tissue *F. nucleatum* DNA and patient mortality (table 2).

In the 1069 colorectal cancer cases, there were 578 deaths, including 315 colorectal cancer-specific deaths, during a median patient follow-up of 10.7 years (IQR: 7.0–15.8) for censored cases. The amount of tissue *F. nucleatum* DNA was associated with higher colorectal cancer-specific mortality in univariable (p for trend=0.023) and multivariable Cox regression analyses (p for trend=0.020). Compared with *F. nucleatum*-negative cases, multivariable HRs for colorectal cancer-specific mortality in *F. nucleatum*-low cases and *F. nucleatum*-high cases were 1.25 (95% CI 0.82 to 1.92) and 1.58 (95% CI 1.04 to 2.39), respectively. In Kaplan-Meier analysis, a higher amount of tissue *F. nucleatum* DNA was associated with shorter colorectal cancer-specific survival (p=0.023 by the log-rank test for trend; figure 1A).

In a secondary analysis of overall mortality as an outcome, the amount of tissue *F. nucleatum* DNA was not significantly associated with overall mortality (p for trend=0.99; table 2: p=0.50 by the log-rank test for trend; figure 1B).

Considering that disease stage and tumour differentiation may be on the causal pathway from the amount of tissue *F. nucleatum* DNA to shorter survival, we also used the multivariable Cox regression model that did not include disease stage or tumour differentiation in a further secondary analysis, and observed a significant association of the amount of tissue *F. nucleatum* DNA with higher colorectal cancer-specific mortality (p for trend=0.0001; see online supplementary table S1).

Tissue *F. nucleatum* in relation to other features in colorectal cancer

As shown in table 1, a higher amount of tissue *F. nucleatum* DNA was associated with proximal tumour location, higher pT

Table 1 Characteristics according to the amount of *Fusobacterium nucleatum* DNA in colorectal cancer tissue

Characteristic*	All patients (n=1069)	The amount of *F. nucleatum* DNA in colorectal cancer tissue			p Value†
		Negative (n=935)	Low (n=67)	High (n=67)	
Mean age±SD (year)	69.3±8.8	69.2±8.8	71.1±9.0	68.8±8.3	0.21
Sex					0.36
Men	449 (42%)	400 (43%)	26 (39%)	23 (34%)	
Women	620 (58%)	535 (57%)	41 (61%)	44 (66%)	
Year of diagnosis					0.016
Prior to 1995	351 (33%)	322 (34%)	12 (18%)	17 (25%)	
1996 to 2000	298 (28%)	260 (28%)	18 (27%)	20 (30%)	
2001 to 2008	420 (39%)	353 (38%)	37 (55%)	30 (45%)	
Family history of colorectal carcinoma in a first-degree relative					0.27
Absent	857 (80%)	745 (80%)	59 (88%)	53 (80%)	
Present	208 (20%)	187 (20%)	8 (12%)	13 (20%)	
Tumour location					0.001
Caecum	178 (17%)	145 (15%)	13 (20%)	20 (30%)	
Ascending to transverse colon	346 (32%)	296 (32%)	23 (34%)	27 (41%)	
Splenic flexure to sigmoid	311 (29%)	287 (31%)	12 (18%)	12 (18%)	
Rectosigmoid and rectum	231 (22%)	205 (22%)	19 (28%)	7 (11%)	
pT stage (depth of tumour invasion)					0.0008
pT1 (submucosa)	99 (10%)	93 (11%)	1 (1.6%)	5 (8.2%)	
pT2 (muscularis propria)	205 (21%)	189 (22%)	12 (19%)	4 (6.6%)	
pT3 (subserosa)	620 (63%)	532 (62%)	41 (66%)	47 (77%)	
pT4 (serosa or other organs)	57 (5.8%)	44 (5.1%)	8 (13%)	5 (8.2%)	
pN stage (number of positive lymph nodes)					0.84
pN0 (0)	602 (63%)	531 (64%)	35 (58%)	36 (61%)	
pN1 (1–3)	211 (22%)	182 (22%)	16 (27%)	13 (22%)	
pN2 (≥4)	138 (15%)	119 (14%)	9 (15%)	10 (17%)	
AJCC disease stage					0.003
I	241 (25%)	225 (26%)	9 (15%)	7 (11%)	
II	325 (33%)	274 (32%)	23 (37%)	28 (45%)	
III	278 (28%)	239 (28%)	25 (40%)	14 (23%)	
IV	133 (14%)	115 (14%)	5 (8.1%)	13 (21%)	
Tumour differentiation					<0.0001
Well to moderate	965 (90%)	862 (92%)	55 (83%)	48 (72%)	
Poor	102 (9.6%)	72 (7.7%)	11 (17%)	19 (28%)	
MSI status					<0.0001
MSI-low/MSS	858 (84%)	780 (87%)	42 (66%)	36 (55%)	
MSI-high	165 (16%)	114 (13%)	22 (34%)	29 (45%)	
MLH1 hypermethylation					<0.0001
Absent	844 (86%)	759 (89%)	48 (79%)	37 (60%)	
Present	134 (14%)	96 (11%)	13 (21%)	25 (40%)	
CIMP status					<0.0001
Low/negative	800 (82%)	716 (84%)	48 (79%)	36 (58%)	
High	178 (18%)	139 (16%)	13 (21%)	26 (42%)	
BRAF mutation					0.0009
Wild type	866 (84%)	771 (86%)	50 (78%)	45 (68%)	
Mutant	165 (16%)	130 (14%)	14 (22%)	21 (32%)	
KRAS mutation					0.44
Wild type	566 (57%)	499 (57%)	29 (50%)	38 (61%)	
Mutant	423 (43%)	370 (43%)	29 (50%)	24 (39%)	
PIK3CA mutation					0.88
Wild type	813 (84%)	713 (84%)	48 (83%)	52 (83%)	
Mutant	157 (16%)	136 (16%)	10 (17%)	11 (17%)	
Mean LINE-1 methylation level (%)±SD	63.3±10.0	63.1±10.0	64.8±10.7	65.2±8.9	0.11

*Percentage indicates the proportion of cases with a specific clinical, pathological or tumour molecular feature according to the amount of *F. nucleatum* DNA in colorectal cancer tissue. There were cases which had missing values for any of the characteristics except for age, sex and year of diagnosis.
†To assess associations between the ordinal categories (negative, low and high) of the amount of *F. nucleatum* DNA in colorectal cancer tissue and categorical variables, Fisher's exact test was performed. To compare mean age and mean LINE-1 methylation levels, an analysis of variance was performed. We adjusted two-sided α level to 0.003 (=0.05/16) by simple Bonferroni correction for multiple hypothesis testing.
AJCC, American Joint Committee on Cancer; CIMP, CpG island methylator phenotype; LINE-1, long interspersed nucleotide element-1; MSI, microsatellite instability; MSS, microsatellite stable.

Table 2 The amount of *Fusobacterium nucleatum* DNA in colorectal cancer tissue and patient mortality

The amount of *F. nucleatum* DNA	No. of cases	Colorectal cancer-specific mortality			Overall mortality		
		No. of events	Univariable HR (95% CI)	Multivariable stage-stratified HR (95% CI)*	No. of events	Univariable HR (95% CI)	Multivariable stage-stratified HR (95% CI)*
Negative	935	265	1 (reference)	1 (reference)	511	1 (reference)	1 (reference)
Low	67	24	1.31 (0.86 to 2.00)	1.25 (0.82 to 1.92)	32	1.01 (0.70 to 1.44)	0.84 (0.59 to 1.21)
High	67	26	1.51 (1.01 to 2.26)	1.58 (1.04 to 2.39)	35	1.14 (0.81 to 1.61)	1.08 (0.76 to 1.52)
p for trend†			0.023	0.020		0.50	0.99

*The multivariable stage-stratified Cox regression model initially included sex, age, year of diagnosis, family history of colorectal cancer in parent or sibling, tumour location, microsatellite instability, CpG island methylator phenotype, *KRAS*, *BRAF* and *PIK3CA* mutations, and long interspersed nucleotide element-1 (LINE-1) methylation level. A backward elimination with a threshold of p=0.05 was used to select variables in the final models.
†Test for a linear trend was conducted across the ordinal categories (negative (0), low (1), and high (2)) of the amount of *F. nucleatum* DNA in colorectal cancer tissue as a continuous variable in the Cox regression model.

stage, poor tumour differentiation, MSI-high, *MLH1* hypermethylation, CIMP-high and *BRAF* mutation (p≤0.001 with the adjusted α level of 0.003 for multiple hypothesis testing).

As an exploratory analysis, we examined associations of tissue *F. nucleatum* DNA with pT stage, pN stage and M stage (table 3).

The amount of tissue *F. nucleatum* DNA was associated with higher pT stage in univariable (p for trend=0.0003) and multivariable ordinal logistic regression analyses (p for trend=0.0007). The association of tissue *F. nucleatum* DNA with pN or M stage was not statistically significant (p for trend ≥0.029 with the adjusted α level of 0.003; table 3).

Table 4 shows the distribution of colorectal cancer cases according to combined MSI/CIMP/*BRAF* status.

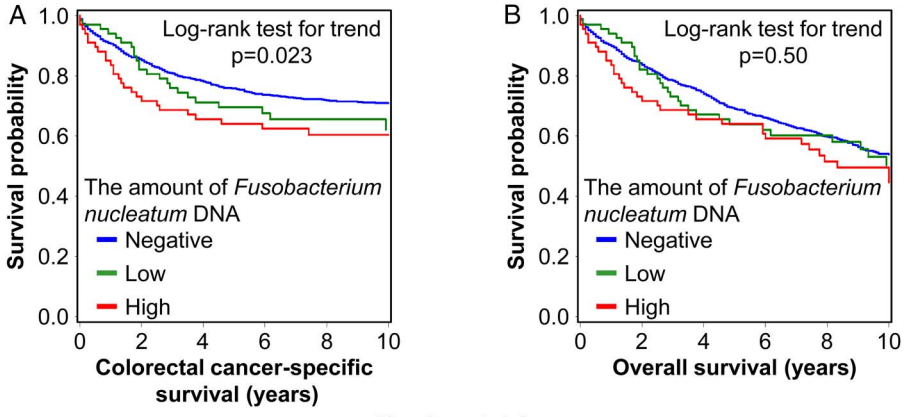
As an exploratory analysis, we performed multivariable ordinal logistic regression analysis to assess independent associations of MSI, CIMP and *BRAF* mutation status with the amount of tissue *F. nucleatum* DNA (table 5).

The amount of tissue *F. nucleatum* DNA was associated with MSI-high (multivariable OR, 5.22; 95% CI 2.86 to 9.55), independent of CIMP and *BRAF* mutation status. In contrast, CIMP or *BRAF* mutation was not associated with *F. nucleatum* after adjusting for MSI status.

DISCUSSION

We conducted this study to test the hypothesis that a higher amount of tissue *F. nucleatum* might be associated with worse clinical outcome in colorectal cancer. Using the database of the 1069 colorectal carcinoma cases in the two US nationwide prospective cohort studies, we observed the association between the amount of tissue *F. nucleatum* DNA and higher colorectal cancer-specific mortality.

Recent studies have provided mechanistic insights into the relationship between *F. nucleatum* and colorectal tumour progression. *F. nucleatum* expresses the virulence factor FadA on the bacterial cell surface, which has been shown to activate the WNT signalling pathway in colorectal carcinoma cells and promote colorectal tumour growth.[18] *F. nucleatum* may inhibit T cell-mediated immune responses against colorectal tumours in the $Apc^{Min/+}$ mouse model.[16 19] Our recent study has shown an inverse association between the amount of tissue *F. nucleatum* DNA and $CD3^+$ T cell density in colorectal cancer.[21] In the present study, a higher amount of tissue *F. nucleatum* DNA was associated with a higher pT stage and worse clinical outcome. These lines of evidence together with the findings from our current study support the hypothesis that *F. nucleatum*-high colorectal cancers may represent a more biologically aggressive cancer subtype. In light of possible roles of *F. nucleatum* in downregulating T cell-mediated antitumour immune responses and in promoting colorectal tumour progression, future investigations may be warranted to explore potential influence of tissue *F. nucleatum* on efficacy of the T cell-based immunotherapies for colorectal cancer.



Number at risk

The amount of *Fusobacterium nucleatum* DNA	Year					
	0	2	4	6	8	10
Negative	935	782	670	546	446	350
Low	67	55	43	34	27	18
High	67	48	43	36	25	18

Figure 1 Kaplan-Meier curves for colorectal cancer-specific survival (A) and overall survival (B) according to the amount of *Fusobacterium nucleatum* DNA in colorectal cancer tissue. The p value was calculated by the log-rank test for trend (two-sided). The table (bottom) shows the number of patients who remained alive and at risk of death at each time point after the diagnosis of colorectal cancer.

Table 3 Association of the amount of *Fusobacterium nucleatum* DNA in colorectal cancer tissue with each component of the American Joint Committee on Cancer staging system

	Univariable OR (95% CI)	Multivariable OR (95% CI)*
Model for pT stage (n=981, as an ordinal outcome variable (pT1 vs pT2 vs pT3 vs pT4))		
The amount of *F. nucleatum* DNA		
Negative	1 (reference)	1 (reference)
Low	2.22 (1.27 to 3.90)	2.41 (1.37 to 4.24)
High	2.24 (1.27 to 3.95)	2.02 (1.14 to 3.56)
p for trend†	0.0003	0.0007
Model for pN stage (n=951, as an ordinal outcome variable (pN0 vs pN1 vs pN2))		
The amount of *F. nucleatum* DNA		
Negative	1 (reference)	1 (reference)
Low	1.21 (0.72 to 2.04)	1.48 (0.86 to 2.52)
High	1.15 (0.68 to 1.94)	1.68 (0.96 to 2.92)
p for trend†	0.45	0.029
Model for M stage (n=977, as a binary outcome variable (M0 vs M1))		
The amount of *F. nucleatum* DNA		
Negative	1 (reference)	1 (reference)
Low	0.56 (0.22 to 1.43)	0.81 (0.31 to 2.11)
High	1.70 (0.90 to 3.24)	2.33 (1.16 to 4.69)
p for trend†	0.32	0.045

*The logistic regression analysis model initially included age, sex, year of diagnosis, family history of colorectal carcinoma in parent or sibling, tumour location, microsatellite instability, CpG island methylator phenotype, *KRAS*, *BRAF* and *PIK3CA* mutations, and long interspersed nucleotide element-1 (LINE-1) methylation level. A backward stepwise elimination with a threshold of p=0.05 was used to select variables in the final models.
†Test for a linear trend was conducted across the ordinal categories (negative (0), low (1), and high (2)) of the amount of *F. nucleatum* DNA in colorectal cancer tissue as a continuous variable in the logistic regression model for pT stage (an ordinal outcome variable), pN stage (an ordinal outcome variable) or M stage (a binary outcome variable). We adjusted two-sided α level to 0.003 for multiple hypothesis testing.

Table 5 Ordinal logistic regression analysis to assess independent associations of MSI, CIMP and *BRAF* mutation status with the amount of *Fusobacterium nucleatum* DNA in colorectal cancer tissue

Model for the amount of *F. nucleatum* DNA (n=953, as an ordinal outcome variable (negative vs low vs high))	Univariable OR (95% CI)	Multivariable OR (95% CI)*
MSI-high (vs MSI-low/MSS)	4.53 (3.04 to 6.75)	5.22 (2.86 to 9.55)
CIMP-high (vs CIMP-low/negative)	2.51 (1.65 to 3.80)	0.71 (0.35 to 1.44)
BRAF mutant (vs wild type)	2.23 (1.46 to 3.42)	1.14 (0.62 to 2.10)

*In addition to MSI, CIMP and *BRAF* mutation status, the multivariable ordinal logistic regression analysis model initially included age, sex, year of diagnosis, family history of colorectal carcinoma in parent or sibling, tumour location, *KRAS* and *PIK3CA* mutations and LINE-1 methylation level. A backward stepwise elimination with a threshold of p=0.05 was used to select variables in the final models, and the 'year of diagnosis' variable remained in the final model.
CIMP, CpG island methylator phenotype; MSI, microsatellite instability; MSS, microsatellite stable.

Colorectal cancers develop through the accumulation of genetic and epigenetic alterations, influenced by microbial and other environmental exposures and host responses to the exposures.[34–38] In the current study, a higher amount of tissue *F. nucleatum* DNA was associated with key tumour molecular features of colorectal cancer, including MSI-high, CIMP-high, LINE-1 hypomethylation and *BRAF* mutation, which have been associated with clinical outcome in colorectal cancer.[39–46] By using over 1000 human colorectal carcinoma cases, to our knowledge we provided the first evidence that supports the prognostic significance of the amount of *F. nucleatum* DNA in colorectal cancer tissue, independent of clinical, pathological and major tumour molecular features. In addition, our current study could demonstrate that tissue *F. nucleatum* was associated with MSI-high, but not with CIMP-high or *BRAF* mutation in multivariate analysis that adjusted for each other.

We acknowledge limitations of our study. First, the data on cancer recurrence were limited in the two cohorts. However, colorectal cancer-specific mortality is a reasonable cancer-specific outcome in the current study, which used the population-based data of long-term patient follow-up, since median survival for recurrent (local or metastatic) colorectal cancer was approximately 10–20 months during much of the time period of this study.[47] Second, the data on cancer treatment were limited. However, it is unlikely that the distribution of chemotherapy use could substantially differ according to the amount of tissue *F. nucleatum* DNA, because the data on tissue *F. nucleatum* DNA were not available for treatment decisions.

Strengths of this study include the use of our molecular pathological epidemiology[48–51] database (of over 1000 colorectal carcinoma cases in the two US nationwide, prospective cohort studies), which integrated epidemiological exposures, clinicopathological features, key tumour molecular features and tissue *F. nucleatum* DNA in colorectal carcinoma. Importantly, our colorectal cancer specimens were derived from a large number of hospitals in diverse settings across the US, which increases the generalisability of our findings. In addition, the

Table 4 The amount of *Fusobacterium nucleatum* DNA in colorectal cancer tissue in relation to combined MSI/CIMP/*BRAF* status

Combined MSI/CIMP/*BRAF* status				The amount of *F. nucleatum* DNA		
MSI status	CIMP status	*BRAF* mutation	All patients (n=953)	Negative (n=833)	Low (n=59)	High (n=61)
High	High	Mutant	83	56 (67%)	10 (12%)	17 (21%)
High	High	Wild type	37	29 (78%)	2 (5.4%)	6 (16%)
High	Low/negative	Mutant	3	1 (33%)	1 (33%)	1 (33%)
High	Low/negative	Wild type	32	21 (66%)	6 (19%)	5 (15%)
MSI-low/MSS	High	Mutant	29	27 (93%)	0	2 (6.9%)
MSI-low/MSS	High	Wild type	27	25 (93%)	1 (3.7%)	1 (3.7%)
MSI-low/MSS	Low/negative	Mutant	40	38 (95%)	1 (2.5%)	1 (2.5%)
MSI-low/MSS	Low/negative	Wild type	702	636 (91%)	38 (5.4%)	28 (4.0%)

Percentage indicates the proportion of *F. nucleatum*-negative, *F. nucleatum*-low or *F. nucleatum*-high cases among all cases with a given specific combined MSI/CIMP/*BRAF* status.
CIMP, CpG island methylator phenotype; MSI, microsatellite instability; MSS, microsatellite stable.

sample size and comprehensiveness of the colorectal cancer database enabled us to assess the prognostic significance of tissue *F. nucleatum* DNA, controlling for potential confounders.

In conclusion, the amount of tissue *F. nucleatum* DNA was associated with higher colorectal cancer-specific mortality. These findings need to be validated in additional populations, as analytical and clinical validations are important to implement clinical use of tumour tissue biomarkers.[52] Upon validation, *F. nucleatum* DNA in colorectal carcinoma tissue may serve as a prognostic biomarker. In addition, our population-based data may provide insights for future studies to develop strategies for colorectal cancer prevention and treatment through targeting the microbiota.

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