

ORIGINAL RESEARCH—CLINICAL

Fusobacterium nucleatum Enrichment in Colorectal Tumor Tissue: Associations With Tumor Characteristics and Survival Outcomes

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BACKGROUND AND AIMS: *Fusobacterium nucleatum* (*Fn*) is linked to colorectal cancer (CRC) etiology and survival. We hypothesized that CRC tumor attributes and survival are associated with the amount and presence of *Fn* in tumors. **METHODS:** *Fn* abundance was measured via droplet digital polymerase chain reaction in patient-matched CRC tumor and normal tissue samples from 859 Puget Sound CRC Cohort participants. *Fn* enrichment was defined as the continuous difference in normalized abundance between patient-matched tumor and normal tissue samples. *Fn* presence in tumor was classified categorically as not present, low, or high, regardless of *Fn* status in matched normal tissue. Associations of *Fn* enrichment and presence with tumor site, stage, DNA mismatch repair (MMR) status, CpG island methylator phenotype (CIMP) status, *BRAF* and *KRAS* mutation status, and molecular subtypes based on combinations of tumor markers were assessed using logistic regression. Associations of *Fn* enrichment and presence with CRC survival was estimate with Cox regression. **RESULTS:** *Fn* was present in 20% of tumor tissues and 10% of normal tissues, with higher average abundance in tumors. High *Fn* presence was independently associated with deficient MMR (dMMR) status and in the context of molecular subtypes for type 1 tumors (dMMR, CIMP-high, *BRAF*-mutated) and type 5 tumors (dMMR, CIMP-low or negative, *BRAF*-wildtype). *Fn* enrichment was associated with type 5 and type 2 tumors (proficient MMR, CIMP-high, *BRAF*-mutated). *Fn* enrichment and presence were associated with poorer CRC survival, with some suggestion that associations differed by MMR status. **CONCLUSION:** Detectable *Fn* in CRC tissue is associated with certain CRC tumor attributes and survival; however, associations may vary based on *Fn* definition.

Keywords: *Fusobacterium*; Intratumoral Microbiome; Tumor Microenvironment; CRC; PSCCC

human gut,² growing evidence implicates this genus, particularly *Fusobacterium nucleatum* (*Fn*), in the etiology and progression of colorectal cancer (CRC).^{3–7}

Since Castellarin et al. first described an overabundance of *Fn* in CRC relative to adjacent normal tissue in 2012,³ several studies have provided further support that this species is enriched in a subset of colorectal tumors.^{8–12} In particular, the presence of *Fn* is more common in colorectal tumors arising in the proximal colon,^{10,13,14} and in tumors that exhibit deficient mismatch repair (dMMR) or high microsatellite instability (MSI),^{13,15} the CpG island methylator phenotype (CIMP),^{13,16,17} and somatic *BRAF* mutations.^{13,15} The presence of these bacteria in CRC are also associated with poorer survival and more advanced stage at diagnosis.^{8,13,15,18–20} Using data from longitudinal studies, Mima et al. suggested that these associations with survival¹³ and with tumor site¹³ are most pronounced for those with colorectal tumors exhibiting higher quantified levels of *Fn* in tumor.

Despite growing evidence as to the significance of *Fn* in CRC, most studies focused on the epidemiology of these bacteria in CRC have been limited by small sample size or by methodological constraints. Using data and biospecimens from the large population-based Puget Sound Colorectal Cancer Cohort (PSCCC), we examined the relationship of *Fn*

Abbreviations used in this paper: CIMP, CpG island methylator phenotype; CRC, colorectal cancer; CI, confidence interval; dMMR, deficient mismatch repair; FFPE, formalin-fixed paraffin-embedded; *Fn*, *Fusobacterium nucleatum*; MSI, microsatellite instability; MMR, mismatch repair; OR, odds ratio; pMMR, proficient mismatch repair; PSCCC, Puget Sound Colorectal Cancer Cohort.

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Introduction

Bacteria of the genus *Fusobacterium* are common residents of the human oral microbiome.¹ Although *Fusobacterium* spp. are found infrequently in the healthy

status in colorectal tumor tissue with other prognostically relevant colorectal tumor markers, established molecular subtypes, and survival.

Methods and Materials

Study Population

The PSCCC is comprised of study participants from the Seattle site of the Colon Cancer Family Registry²¹ and includes additional participants from a parallel extension study.²² The Seattle site of the Colon Cancer Family Registry enrolled study participants who were diagnosed with incident invasive CRC between 1998 and 2007 while residing in western Washington State and who were between the ages of 20 and 74 years at the time of diagnosis. Additional criteria for the extension study were female sex and newly diagnosed with invasive CRC between 1998 and 2002. Eligible prospective participants for the PSCCC were identified through the Surveillance, Epidemiology, and End Results cancer registry serving the Seattle-Puget Sound region.

Inclusion was further limited to English speakers with available telephone numbers and baseline epidemiologic questionnaire completion ($N = 2264$). The questionnaire included information regarding demographics, lifestyle, medical history, and family history of cancer. The analysis was further restricted to individuals with DNA that could be extracted from matched tumor and normal colorectal tissue ($N = 898$).

All study participants included in the present analysis provided informed consent for the collection and analysis of their study materials. This study was approved by the institutional review board of the Fred Hutchinson Cancer Center (IR#8650).

Vital Status and Clinicopathologic Factors

Information regarding the vital status and cause and approximate date of death were obtained from the cancer registry via regular linkage to the National Death Index. Vital status information was updated in May 2022. Deaths classified as International Classification of Disease 10 codes C18.0-C20.0 or C26.0 were attributed to CRC. Tumors located in the cecum through the transverse colon were grouped as proximal colon cancers (ICD-O-3 codes C180, C182, C183, and C184). Tumors in the splenic flexure through the sigmoid colon were classified as distal colon cancers (C185, C186, C187), while those in the rectosigmoid junction (C199) and rectum (C209) were grouped together as rectal cancers. Tumor stage at diagnosis was classified according to Surveillance, Epidemiology, and End Results conventions as localized, regional, or distant stage.

Tumor Collection, Tumor Markers, and Tumor Subtype Classification

Formalin-fixed paraffin-embedded (FFPE) diagnostic tumor tissue specimens were obtained from treating institutions for consenting study participants, as were FFPE tissue specimens from tumor-adjacent normal tissue.

Somatic DNA extracted from tumor tissue specimens was used to test for several established tumor markers, including MSI or MMR deficiency status, somatic *BRAF* c.1799T>A

(p.V600E) and *KRAS* mutation status (in codons 12–13), and CIMP status. Methods for collecting data on these tumor markers have been described elsewhere.²³ MMR or MSI status was dichotomized as MMR deficient or MSI-high (dMMR) vs MMR proficient or not MSI-high (pMMR). CIMP status was dichotomized as CIMP-high vs CIMP-low/negative. *BRAF* and *KRAS* mutation status were each dichotomized as mutated vs not mutated.

Consistent with previously suggested²⁴ and evaluated²³ molecular classifications, we defined tumor subtypes as follows: 1) “type 1” (ie, dMMR, CIMP-high, *BRAF*-mutated, not *KRAS* mutated); 2) “type 2” (ie, pMMR, CIMP-high, *BRAF*-mutated, not *KRAS* mutated); 3) “type 3” (ie, pMMR, CIMP-low or neg, not *BRAF*-mutated, *KRAS*-mutated); 4) “type 4” (ie, pMMR, not CIMP-high, not *BRAF*-mutated, not *KRAS*-mutated); and 5) “type 5” (ie, dMMR, not CIMP-high, not *BRAF*-mutated, not *KRAS* mutated).

Bacterial DNA Extraction and *Fn* Testing

A description of the bacterial DNA extraction process and the assay used to determine the presence and extent of *Fn* in tissues is included in the [Supplemental Materials](#) section.

Fn enrichment and Presence

Testing for *Fn* in both tumor and matched normal tissue samples from each study participant allowed us to define *Fn* status based not only on the presence of these bacteria in tumor tissue but also on the extent to which these bacteria were specifically enriched in tumor tissue relative to matched adjacent normal tissue. Thus, we considered 2 operationalizations of *Fn* status: enrichment and presence. Enrichment was defined as the continuous difference in normalized *Fn* counts in tumor vs in matched normal tissue (where positive values indicated that the normalized count of *Fn* in tumor was higher than that in matched normal tissue). *Fn* presence in tumor was classified categorically as not present, low (normalized *Fn* counts in tumor >0 but less than the median level among those positive for *Fn*), or high (normalized *Fn* counts in tumor greater than or equal to the median), without regard to *Fn* status in matched normal tissue.

Statistical Analysis

We conducted logistic regression analyses to examine associations of *Fn* enrichment and presence with tumor characteristics, individual tumor markers (MMR, CIMP, *BRAF* and *KRAS* mutation status), and molecular subtypes based on tumor marker combinations. *Fn* enrichment was evaluated as a continuous variable and was transformed to estimate the change associated with a difference of 0.10 counts in *Fn*; *Fn* presence was examined as a categorical variable. Analyses were adjusted for age at CRC diagnosis, sex, and all other analyzed tumor attributes. Models for *Fn* enrichment were further adjusted for normalized normal tissue *Fn* abundance.

Cox proportional hazards regression were used to evaluate the associations of *Fn* enrichment and presence with CRC survival and we explored whether MMR status modified the association. Time was measured in days since diagnosis, with left truncation to account for the time between diagnosis and enrollment (median = 7.2 months). Participants alive at their

last vital status assessment were censored at that date; persons who died due to causes other than CRC were censored at the time of death. Proportional hazards assumptions were assessed by testing for a nonzero slope of the scaled Schoenfeld residuals on ranked failure times.

Missing Data

Individuals with missing data for >2 tumor markers were excluded from the analysis (N = 39). Multiple imputation was used to approximate tumor subtype classification status for cases with missing data for 1 (N = 269) or 2 (N = 33) of the 4 tumor markers used in subtype classification.^{25,26} The imputation model included variables for tumor markers, stage at diagnosis, tumor site, sex, age at diagnosis, diagnosis year, body mass index, height, smoking history, use of nonsteroidal anti-inflammatory drugs at diagnosis, history of endoscopic screening before diagnosis, time from diagnosis to interview, *Fn* status, censoring indicators, and analysis time. Iterative rounds of imputation (N = 100) were performed using the *Amelia II* package²⁷ in R version 4.1.2.²⁸ Tumor subtype classifications were thus determined based on assayed and imputed tumor markers as necessary.

Results

Study participants' mean age at diagnosis was 57 years and 55% were female. About 45% of individuals had tumors located in the proximal colon and 57% had regional stage tumors. Participants identified as having low or high *Fn* presence in tumor were more likely to be female than male

($P = .01$) than were those with no *Fn* present in tumor (67% and 63% vs 52%, respectively). In addition, compared to those with no detected *Fn* in tumor, participants with high *Fn* presence were more likely to have tumors located in the proximal colon (66% vs 41%, $P < .001$), diagnosed at regional stage (65% vs 56%, $P = .5$), and to have tumors exhibiting dMMR (44% vs 13%, $P < .001$), CIMP-high status (40% vs 16%, $P < .001$), and somatic *BRAF* c.1799T>A (p.V600E) mutations (26% vs 11%, $P < .001$) (Table 1). Table 2 shows the distribution of normalized *Fn* counts by tissue type and MMR status.

After multivariable adjustment, *Fn* enrichment and presence were not significantly associated with tumor site, stage at diagnosis, CIMP-high, or *BRAF* and *KRAS* mutation status (Table 3). High *Fn* presence was positively associated with dMMR status (odds ratio (OR) vs absent = 3.47; 95% confidence interval (CI): 1.81–6.66); however, no significant association was observed between *Fn* enrichment and dMMR status (OR = 1.04, 95% CI: 0.96–1.13).

Consistent with findings of association with dMMR status after adjusting for other tumor markers, high *Fn* presence was associated with types 1 and 5 tumor classification (OR = 5.33; 95% CI: 2.25–12.62, OR = 3.20; 95% CI: 1.15–8.88) (Table 4). Both subtypes were also suggestively associated with *Fn* enrichment (OR = 1.23; 95% CI: 1.00–1.51 and OR 1.29; 95% CI 1.05–1.59, respectively). Type 2 tumor classification was associated with *Fn* enrichment (OR 1.30; 95% CI 1.06–1.60), but not *Fn* presence.

Table 1. Characteristics of Colorectal Cancer Patients by *Fn* Presence (N = 859)

Characteristic	Overall N = 859 ^a	Not present N = 683 ^a	Low N = 90 ^a	High N = 86 ^a	<i>P</i> ^b
<i>Fn</i> enrichment	0.000 (0.000, 0.000)	0.000 (0.000, 0.000)	0.004 (0.001, 0.010)	0.079 (0.041, 0.190)	<.001
Age at diagnosis (y) (standard deviation)	57 (12)	57 (12)	58 (12)	58 (13)	.5
Sex					.01
Male	389 (45%)	327 (48%)	30 (33%)	32 (37%)	
Female	470 (55%)	356 (52%)	60 (67%)	54 (63%)	
Tumor site					<.001
Proximal	387 (45%)	279 (41%)	51 (57%)	57 (66%)	
Distal	244 (29%)	201 (30%)	27 (30%)	16 (19%)	
Rectal	223 (26%)	199 (29%)	11 (12%)	13 (15%)	
Missing	5	4	1	0	
Stage at diagnosis					.5
Local	260 (31%)	212 (32%)	27 (31%)	21 (24%)	
Regional	479 (57%)	375 (56%)	48 (55%)	56 (65%)	
Distant	102 (12%)	80 (12%)	13 (15%)	9 (10%)	
Missing	18	16	2	0	
dMMR	152 (18%)	86 (13%)	30 (36%)	36 (44%)	<.001
Missing	30	20	6	4	
CIMP-high	124 (20%)	78 (16%)	20 (29%)	26 (40%)	<.001
Missing	241	200	20	21	
<i>BRAF</i> -mutated	112 (13%)	73 (11%)	17 (20%)	22 (26%)	<.001
Missing	14	11	3	0	
<i>KRAS</i> -mutated	256 (32%)	209 (33%)	27 (31%)	20 (25%)	.4
Missing	48	40	2	6	

^aMean (SD) or n (%).

^bKruskal-Wallis rank sum test; Pearson's Chi-squared test.

Table 2. Distribution of Normalized *Fn* Counts by Tissue type and MMR Status (N = 829)

	Minimum	Quartile 1	Median	Mean	Quartile 3	Max
dMMR						
Normal	<0.000001	<0.000001	<0.000001	0.007166	<0.000000	0.608800
Tumor	<0.000001	<0.000001	<0.000001	0.08758	<0.02092	3.13230
pMMR						
Normal	<0.000001	<0.000001	<0.000001	0.002333	<0.000000	0.26650
Tumor	<0.000001	<0.000001	<0.000001	0.02091	<0.000000	5.06310

Table 3. Association Between *Fn* enrichment and Tumor Attributes in Colorectal Cancer Patients (N = 859)

Outcome: Tumor attributes	Exposure: <i>Fn</i> enrichment ^a			Exposure: <i>Fn</i> presence (vs Negative) ^b					
	OR ^b	95% CI	<i>P</i> value	Low			High		
				OR ^{3F}	95% CI	<i>P</i> value	OR ^c	95% CI	<i>P</i> value
Tumor site									
Right-Sided	Ref	-	-	Ref	-	-	Ref	-	-
Left-Sided	1.00	(0.93, 1.08)	.98	1.03	(0.59, 1.81)	.92	0.69	(0.36, 1.33)	.27
Rectal	0.81	(0.55, 1.2)	.30	0.50	(0.24, 1.06)	.07	0.68	(0.32, 1.42)	.30
Stage at diagnosis									
Local	Ref	-	-	Ref	-	-	Ref	-	-
Regional	1.08	(0.97, 1.2)	.17	1.06	(0.63, 1.78)	.83	1.67	(0.95, 2.92)	.07
Distant	0.91	(0.61, 1.36)	.65	1.70	(0.8, 3.6)	.17	1.58	(0.66, 3.78)	.31
dMMR	1.04	(0.96, 1.13)	.32	3.42	(1.74, 6.72)	<.001	3.47	(1.81, 6.66)	<.001
CIMP-high	1.08	(0.98, 1.18)	.12	0.94	(0.38, 2.35)	.90	1.81	(0.8, 4.08)	.15
BRAF-mutated	0.97	(0.91, 1.04)	.39	0.86	(0.31, 2.41)	.77	0.61	(0.25, 1.51)	.28
KRAS-mutated	0.95	(0.83, 1.08)	.44	1.11	(0.66, 1.89)	.69	0.87	(0.49, 1.57)	.65

^a*Fn* enrichment was defined as the continuous difference in *Fn* abundance (normalized to counts of a host housekeeping gene) between patient-matched tumor and normal tissue samples. *Fn* enrichment was evaluated as a continuous variable and evaluated as difference of 0.10 counts (rather than 1.0 counts).

^b*Fn* presence in tumor was classified categorically as negative, low (*Fn* abundance >0 but < median level among those positive for *Fn*), or high (abundance ≥ median).

^cTumor site and stage at diagnosis were assessed using multinomial logistic regression and tumor markers were assessed using binary logistic regression. Models were adjusted for age at diagnosis, sex, all other tumor attributes, and normal tissue *Fn* abundance (*Fn* enrichment analyses only).

Fn enrichment and high *Fn* presence were both significantly associated with poorer CRC survival after multivariable adjustment, including adjustment for tumor markers and tumor site (HR 1.06; 95% CI 1.01–1.11 and HR 1.52; 95% CI 1.02–2.27, respectively). However, in analyses stratified according to MMR status, the association of *Fn* enrichment with survival was observed only among individuals with pMMR tumors (HR 1.10; 95% CI 1.05–1.16) (Table 5). Conversely, the association of *Fn* presence with survival appeared to be strongest among those with dMMR tumors, although this association was not statistically significant.

Discussion

Using data from a population-based cohort of CRC patients, we examined the relationship of *Fn* enrichment and presence in colorectal tumor tissue with prognostically relevant colorectal tumor markers, established molecular

subtypes, and survival. Our findings suggest that *Fn* status in CRC is associated with certain CRC tumor attributes and survival; however, associations varied slightly according to how *Fn* status was defined. In particular, the presence of high levels of *Fn* in colorectal tumors was positively associated with dMMR status, and with dMMR tumor subtypes (ie, types 1 and 5), while enrichment of *Fn* in tumor relative to adjacent normal tissue was not associated with dMMR. Associations of *Fn* status with survival also varied by *Fn* classification metric and by dMMR status. These findings improve our understanding as to the potential mechanisms through which *Fn* may impact the natural history of CRC.

Consistent with our findings, prior studies have suggested that the prevalence of *Fn* enrichment varies by CRC attributes. In particular, *Fn* prevalence has been suggested to be more common in CRC that is dMMR,^{4,6,9,29,30} CIMP-high,^{4,6,9} and BRAF-mutated.^{6,9,29} While we observed differences in the distribution of *Fn* according to all these

Table 4. Associations Between *Fn* enrichment and Presence of *Fn* in Tumor Tissue With Tumor Molecular Subtype in Colorectal Cancer Patients (N = 859)

Outcome: Tumor molecular subtype (vs Type 4) ^a											
Exposure	Nontype		Type 1		Type 2		Type 3		Type 5		
	OR (95% CI) ^d	P value	OR (95% CI) ^d	P value	OR (95% CI) ^d	P value	OR (95% CI) ^d	P value	OR (95% CI) ^d	P value	
<i>Fn</i> enrichment ^b	1.26 (1.03–1.53)	.03	1.23 (1.00–1.51)	.05	1.30 (1.06–1.60)	.01	1.05 (0.83–1.34)	.68	1.29 (1.05–1.59)	.02	
<i>Fn</i> presence ^c											
Negative	Ref	-	Ref	-	Ref	-	Ref	-	Ref	-	
Low	1.19 (0.50–2.84)	.69	3.34 (1.41–7.89)	.01	1.01 (0.25–4.12)	.99	1.19 (0.65–2.18)	.58	2.49 (0.90–6.90)	.08	
High	3.15 (1.49–6.63)	<.001	5.33 (2.25–12.62)	<.001	1.85 (0.46–7.34)	.38	1.04 (0.51–2.12)	.92	3.20 (1.15–8.88)	.03	

^aMolecular subtypes were defined as type 1 (dMMR, CIMP+, BRAF-mutated); type 2 (pMMR, CIMP+, BRAF-mutated); type 3 (pMMR, non-CIMP, BRAF-mutated); type 4 (pMMR, non-CIMP, BRAF- and KRAS-wildtype); and type 5 (dMMR, non-CIMP, BRAF- and KRAS-wildtype).

^b*Fn* enrichment was defined as the continuous difference in *Fn* abundance (normalized to counts of a host housekeeping gene) between patient-matched tumor and normal tissue samples. *Fn* enrichment was evaluated as a continuous variable and evaluated as difference of 0.10 counts (rather than 1.0 counts).

^c*Fn* presence in tumor was classified categorically as negative, low (*Fn* abundance >0 but < median level among those positive for *Fn*), or high (abundance ≥ median).

^dEstimated using multinomial logistic regression adjusted for age at diagnosis, sex, tumor site, and normalized *Fn* abundance in normal tissue (*Fn* enrichment only).

^aMolecular subtypes were defined as type 1 (dMMR, CIMP+, BRAF-mutated); type 2 (pMMR, CIMP+, BRAF-mutated); type 3 (pMMR, non-CIMP, KRAS-mutated); type 4 (pMMR, non-CIMP, BRAF- and KRAS-wildtype); and type 5 (dMMR, non-CIMP, BRAF- and KRAS-wildtype).
^b*Fn* enrichment was defined as the continuous difference in *Fn* abundance (normalized to counts of a host housekeeping gene) between patient-matched tumor and normal tissue samples. *Fn* enrichment was evaluated as a continuous variable and evaluated as difference of 0.10 counts (rather than 1.0 counts).
^c*Fn* presence in tumor was classified categorically as negative, low (*Fn* abundance >0 but < median level among those positive for *Fn*), or high (abundance ≥ median).
^dEstimated using multinomial logistic regression adjusted for age at diagnosis, sex, tumor site, and normalized *Fn* abundance in normal tissue (*Fn* enrichment only).

tumor markers, after accounting for the correlated nature of the markers, only the association of *Fn* presence with dMMR remained independently statistically significant. In the case of the association of *Fn* with dMMR, it is possible that these bacteria may be acting to promote the CRC etiologic pathways that result in dMMR. For instance, lab-based studies have shown the various ways that gut bacteria can promote carcinogenesis, including enhancement of host proinflammatory genes and cytokines, oxidative stress, and via bacteria-derived genotoxins.^{4,5,31–37} In the case of *Fn*, the virulence factor adhesion FadA can attach to and invade colonic epithelial cells and activate β -catenin signaling, leading to increased expression of oncogenes, transcription factors, inflammatory genes, and Wnt signaling.⁵ These effects may disproportionately contribute to a phenotype of dMMR in promoting pathways of colorectal carcinogenesis.

Some studies have also suggested that *Fn* is more commonly found in tumors located in the proximal colon than in distal colon or rectal tumors,^{10,13,17,38} and with declining abundance from the proximal colon to distal colon and rectum,^{20,39,40} which is also consistent with the observed association between *Fn* status and dMMR status. Others have noted a higher prevalence of *Fn* with more advanced stage at diagnosis.^{8,13,15,17–20} However, we did not observe any association of *Fn* enrichment or presence with tumor site or stage at diagnosis after accounting for other tumor attributes (including MMR status).

Consistent with prior work, we found that *Fn* was associated with poorer CRC survival.^{8,13,15,17–20} In addition, we found evidence that the association between *Fn* enrichment and CRC-specific survival may differ according to MMR status, with a stronger association among individuals with pMMR relative to dMMR tumors. It is possible that associations of *Fn* with survival could be mediated by tumor markers. The role of *Fn* in survival also suggests that it can act as a prognostic marker or can inform the development of CRC prevention and treatment strategies.

To our knowledge, this is the first study to examine how *Fn* status is related to molecular subtypes, defined according to previously suggested²⁴ and evaluated²³ molecular classifications, and the first study to examine associations with both *Fn* presence and enrichment. Our finding that *Fn* presence, and to a lesser extent, enrichment, is associated with type 1 and 5 tumors, is consistent with our findings of a strong association between *Fn* and dMMR status. At least one study reported that *Fn* enrichment was associated with consensus molecular subtype 1 tumors¹¹ (described by dMMR, CIMP-high, *BRAF*-mutated, and with high levels of immune infiltration and activation⁴¹). In contrast, type 2 tumors, which differ from type 1 with regard to MMR status only and are suggested to reflect a serrated-like pathway of development with poor prognosis, were associated with *Fn* enrichment, and to a lesser extent, presence. This suggests a potential role of *Fn* in multiple pathways of CRC development.

By incorporating the counts of *Fn* in not only tumor tissue but also adjacent normal tissue, our study was able to

Table 5. Association Between *Fn* enrichment and Presence of *Fn* in Tumor Tissue With CRC-Specific Survival in Colorectal Cancer Patients (N = 859)

Exposure	Overall			dMMR			pMMR		
	HR ^{a,c}	95% CI	P value	HR ^{a,c}	95% CI	P value	HR ^{a,c}	95% CI	P value
<i>Fn</i> enrichment	1.06	(1.01, 1.11)	.01	1.00	(0.88, 1.13)	.99	1.10	(1.05, 1.16)	<.001
<i>Fn</i> presence ^b									
Negative	Ref	-	-	Ref	-	-	Ref	-	-
Low	1.23	(0.81, 1.88)	.32	1.58	(0.45, 5.44)	.44	1.25	(0.79, 1.99)	.32
High	1.52	(1.02, 2.27)	.04	2.90	(0.99, 8.52)	.05	1.43	(0.89, 2.29)	.13

HR, hazard ratio.

^a*Fn* enrichment was defined as the continuous difference in *Fn* abundance (normalized to counts of a host housekeeping gene) between patient-matched tumor and normal tissue samples. *Fn* enrichment was evaluated as a continuous variable and evaluated as difference of 0.10 counts (rather than 1.0 counts).

^b*Fn* presence in tumor was classified categorically as negative, low (*Fn* abundance >0 but < median level among those positive for *Fn*), or high (abundance ≥ median).

^cEstimated using Cox regression adjusted for age, sex, tumor site, CIMP, status; BRAF, and KRAS, mutation status, and normalized *Fn* abundance in normal tissue (*Fn* enrichment only).

untangle whether *Fn* is likely to be acting in only localized tumor regions or more diffusely beyond the tumor margins. Our finding that *Fn* is more commonly present, and at higher abundance, in normal colon tissue among individuals with dMMR vs pMMR tumors further supports the etiologic differences between these tumor types.

Strengths and Limitations

Our study is strengthened by the availability of both tumor and adjacent normal tissue. Most tissue-based studies investigating *Fn* and CRC have only assayed tumor tissue which offers less sensitivity for evaluating tumor-localized alterations to the gut microbial community and is subject to confounding by external effects on the microbiota (eg, diet, germline genetics). Another advantage of our study is the use of a population-based cohort that allows for more sensitive observations of differences in *Fn* status according to tumor attributes relative to previous studies that have relied on small numbers.

Our study is limited by the inability to perform longitudinal analysis which prevents inferences about whether the presence of *Fn* precedes the appearance of studied molecular features in CRC. Several studies have noted *Fn* enrichment in precancerous colorectal lesions,^{17,42} which suggests that the role of these bacteria in CRC etiology may begin at an early stage, but additional work is needed to clarify the temporal relationship between *Fn*, molecular markers, and tumor development. Our ddPCR-based approach to characterizing *Fn* also precluded any spatial analyses as to where in the tumor (or paired normal) tissue these bacteria were localized; some prior studies have suggested that *Fn* clusters into microniches in tumor, with clustering patterns that may be associated with cancer progression.^{43,44} In addition, our study lacked racial and ethnic diversity. Minoritized communities in the US experience elevated burden of CRC⁴⁵ and some research suggests that there may be differences in the composition of

the gut microbiome across racial and ethnic groups,⁴⁶ which underscores the need for representative studies across diverse populations. Lastly, the FFPE-based tissue samples used in this study had varying length of storage and storage conditions which may have introduced some bias into bacterial assessment. However, paired tumor and normal tissues were collected at the same time and were stored under the same conditions, such that analyses of *Fn* enrichment (ie, focused on the difference in normalized abundance within paired tissues) likely mitigates some concerns regarding potential contamination.

Conclusion

In conclusion, using data from a large population-based cohort study, we evaluated the relationship of *Fn* status in colorectal tumor tissue, quantified 2 different ways, with other prognostically relevant colorectal tumor markers, established molecular subtypes, and survival. High levels of *Fn* in colorectal tumors was positively associated with dMMR status and with dMMR tumor subtypes (ie, types 1 and 5). In comparison, enrichment of *Fn* in tumor relative to adjacent normal tissue was not associated with dMMR. Associations of *Fn* status with survival also varied by *Fn* classification metric and by dMMR status. Our study contributes to the growing evidence for the complex relationship of *Fn* with CRC survival and potential heterogeneity across individual tumor markers and established molecular subtypes.

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Conflicts of Interest:

The authors disclose no conflicts.

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Ethical Statement:

This study was approved by the Institutional Review Board of the Fred Hutchinson Cancer Center (IR#8650).

Data Transparency Statement:

Data elements from the Colon Cancer Family Registry are available for public access via the database of Genotypes and Phenotypes (dbGaP) and microbial data can be made available upon reasonable request to the corresponding author (A.I.P.).

Reporting Guidelines:

STROBE.