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ORIGINAL ARTICLE

Prognostic Impact of *Fusobacterium nucleatum* Depends on Combined Tumor Location and Microsatellite Instability Status in Stage II/III Colorectal Cancers Treated with Adjuvant Chemotherapy

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Background: This study aimed to investigate the prognostic impact of intratumoral *Fusobacterium nucleatum* in colorectal cancer (CRC) treated with adjuvant chemotherapy. **Methods:** *F. nucleatum* DNA was quantitatively measured in a total of 593 CRC tissues retrospectively collected from surgically resected specimens of stage III or high-risk stage II CRC patients who had received curative surgery and subsequent oxaliplatin-based adjuvant chemotherapy (either FOLFOX or CAPOX). Each case was classified into one of the three categories: *F. nucleatum*–high, –low, or –negative. **Results:** No significant differences in survival were observed between the *F. nucleatum*–high and –low/negative groups in the 593 CRCs (p = .671). Subgroup analyses according to tumor location demonstrated that disease-free survival was significantly better in *F. nucleatum*–high than in –low/negative patients with non-sigmoid colon cancer (including cecal, ascending, transverse, and descending colon cancers; n = 219; log-rank p = .026). In multivariate analysis, *F. nucleatum* was determined to be an independent prognostic factor in non-sigmoid colon cancers (hazard ratio, 0.42; 95% confidence interval, 0.18 to 0.97; p = .043). Furthermore, the favorable prognostic effect of *F. nucleatum*–high was observed only in a non-microsatellite instability-high (non-MSI-high) subset of non-sigmoid colon cancers (log-rank p = 0.014), but not in a MSI-high subset (log-rank p = 0.844), suggesting that the combined status of tumor location and MSI may be a critical factor for different prognostic impacts of *F. nucleatum* in CRCs treated with adjuvant chemotherapy. **Conclusions:** Intratumoral *F. nucleatum* load is a potential prognostic factor in a non-MSI-high/non-sigmoid/non-rectal cancer subset of stage II/III CRCs treated with oxaliplatin-based adjuvant chemotherapy.

Key Words: Colorectal neoplasms; Fusobacterium; Gastrointestinal microbiome; Prognosis

Accumulating evidence has implicated the gut microbiota as having various roles in carcinogenesis, prognosis, and treatment response of colorectal cancer (CRC).[1-5] Among the gut microbiota, *Fusobacterium nucleatum* has been identified as a specifically enriched species within the tumor tissue of human CRC.[6,7] Although *F. nucleatum* composes a relatively small proportion of the normal intestinal flora, the amount of tumor-invasive *F. nucleatum* was reported to be remarkably increased in a subset of CRC cases.[6-9]

In the colorectal carcinogenesis pathway, the amount of invasive *F. nucleatum* gradually increases from premalignant adenomatous lesions to carcinomas in the large intestine.[9-11] Among the premalignant colorectal lesions, sessile serrated adenomas have been suggested to be closely correlated with *F. nucleatum* enrichment.[9,10] Therefore, it has been suspected that *F. nucleatum* might have roles in the serrated carcinogenesis pathway of the colorectum. However, detailed mechanisms of the increase of invasive *F. nucleatum* abundance and pathobiological implications of *F. nucleatum* in the serrated pathway are unclear. Experimental data indicate that *F. nucleatum* might have carcinogenic roles through the modulation of the E-cadherin/β-catenin signalling pathway and/or promotion of the pro-inflammatory micro-

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environment.[1,2] However, these biological mechanisms cannot fully explain the basis of the association of *F. nucleatum* with the serrated pathway in CRC.

The findings using clinical samples support the suggestion that a high load of intratumoral *F. nucleatum* is associated with various clinicopathological and molecular features of CRC, including right-sided tumor location, poor prognosis, poor response to chemotherapy, low density of $CD3^+$ tumor-infiltrating lymphocytes, high density of tumor-infiltrating macrophages, CpG island methylator phenotype (CIMP), and microsatellite instability (MSI).[3,4,8,12-14] However, these observed associations of *F. nucleatum* in CRC are less robust, since the results were derived from limited study cohorts. Thus, precise clinicopathological and molecular implications of *F. nucleatum*–high CRC need to be elucidated and validated using additional independent data.

Recent studies reported that the gut microbiota is associated with responses to chemotherapy and immunotherapy in solid tumors.[4,5,15-17] Especially, Yu *et al*.[4] reported that *F. nucleatum* can promote chemoresistance in CRC by modulating the Toll-like receptor, micro-RNAs, and autophagy pathways. Based on these results, we designed a study to investigate the prognostic impacts of *F. nucleatum* in CRC patients treated with adjuvant chemotherapy. The amount of intratumoral *F. nucleatum* and its prognostic associations were analyzed in a total of 593 stage III or high-risk stage II CRCs treated with adjuvant FOLFOX (folinic acid/5-fluorouracil plus oxaliplatin) or CAPOX (capecitabine plus oxaliplatin) chemotherapy.

MATERIALS AND METHODS

Case selection

Formalin-fixed, paraffin-embedded (FFPE) tissues of 747 consecutive series of primary CRCs were collected from the pathology archive of Seoul National University Hospital, Seoul, Korea. All the tissues were from surgical specimens of patients who underwent curative surgery and subsequent adjuvant chemotherapy for stage III or high-risk stage II CRC at Seoul National University Hospital from 2005 to 2012. The inclusion criteria for the case selection were age greater than 18 years, adenocarcinoma histology without neuroendocrine or squamous cell component, stage III or high-risk stage II according to pathological staging, complete resection (R0) of the primary tumor with tumor-free resection margins, and the completion of at least six cycles of adjuvant FOLFOX chemotherapy or four cycles of adjuvant CAPOX therapy. The criteria for high-risk stage II were tumor invasion into visceral peritoneum or direct invasion into adjacent organs/structures (pT4), clinically obstruction or perforation, poorly differentiated or undifferentiated histology (G3/G4), lymphovascular invasion, and perineural invasion. The patients who received pre-operative neoadjuvant chemotherapy and/or radiotherapy (especially patients with rectal cancer) and patients with a history of other malignancy within 5 years were excluded. Initially, 747 cases were subjected to quantitative polymerase chain reaction (qPCR) analysis for *F. nucleatum*. Among them, 154 inadequate samples determined by invalid or poor quality results from the qPCR analysis, as described subsequently, were excluded. Finally, a total of 593 CRC cases were analyzed. The Institutional Review Board of our hospital approved this study (IRB No. 1805-018-944). The Institutional Review Board exempted our study from obtaining informed consent from patients because our study was a retrospective, anonymous, tissue-based investigation.

Clinicopathological data

Clinical data, including age, sex, tumor location, and gross tumor type, were collected from electronic medical records. Hematoxylin and eosin-stained tissue slides of each case were independently reviewed by pathologists (J.M.B. and G.H.K.) to evaluate histopathological features, including pT/pN categories, tumor grade, lymphovascular invasion, perineural invasion, and mucinous histology.

qPCR for *F. nucleatum*

Genomic DNA extraction from FFPE tissues of the 747 CRCs and qPCR for *F. nucleatum*, using the 747 tumor DNA samples, were conducted as previously described.[14] In brief, the following primers and probes targeting the 16S rRNA gene DNA sequence of *F. nucleatum* and the reference gene (prostaglandin transporter, PGT), were used: *F. nucleatum* forward primer, 5'-CAACCATTACTTTAACTCTACCATGTTCA-3'; *F. nucleatum* reverse' primer, 5'-GTTGACTTTACAGAAGGAGATTATGTAAAAATC-3'; *F. nucleatum* FAM probe, 5'-GTTGACTTTACAGAAGGAGATTA-3'; PGT forward primer, 5'-ATCCCCAAAGCACCTGGTTT-3'; PGT reverse primer, 5'-AGAGGCCAAGATAGTCCTGGTAA-3'; PGT VIC probe, 5'-CCATCCATGTCCTCATCTC-3'.[14] The PCR conditions were 95°C for 10 minutes followed by 45 cycles of 95°C for 15 seconds, and 60°C for 1 minute.[14] To compare the *F. nucleatum* DNA amounts between tumor DNA samples, the relative values ($2^{-\Delta Ct}$) calculated from the threshold cycle (Ct) values for *F. nucleatum* normalized to PGT were used. The qPCR method was validated using serially-diluted *F. nucleatum* genomic DNA samples (25586D-5;

ATCC, Manassas, VA, USA). The results of the validation analysis are summarized in Supplementary Fig. S1. *F. nucleatum*–positive CRCs were further classified into two subgroups (*F. nucleatum*–high or *F. nucleatum*–low) using a cut-off median value of $2^{-\Delta Ct}$. Among the samples of the initial 747 cases subjected to *F. nucleatum* qPCR analysis, those of 154 cases were determined as failed or inadequate, based on non-evaluable or high Ct values of PGT. Thus, 593 cases were finally included in this study. The qPCR experiment of each sample was performed independently in triplicate.

DNA analyses for MSI, CIMP, *KRAS*, and *BRAF*

Major molecular factors, including MSI, CIMP, and *KRAS*/*BRAF* mutations, in the CRC samples were analyzed as previously described.[18] Genomic DNA of each tumor was isolated from representative FFPE tissue blocks by microdissection. MSI testing was performed by DNA fragment analysis using five microsatellite markers (BAT-25, BAT-26, D5S346, D17S250, and D2S123) according to the Bethesda guideline.[19] MSI status of each case was classified into one of the three categories: MSI-high, MSI-low, and microsatellite stable (MSS). CIMP analysis was carried out by the real-time PCR-based MethyLight assay using eight CIMP markers (*MLH1*, *NEUROG1*, *CRABP1*, *CACNA1G*, *CDKN2A*, *IGF2*, *SOCS1*, and *RUNX3*) as previously described.[18] CIMP status of each case was classified into one of the three categories: CIMP-high, CIMP-low, and CIMP-negative. Mutational status of *KRAS* exon 2 codons 12 and 13 and *BRAF* exon 15 codon 600 were examined by Sanger sequencing.

Statistical analyses

All statistical analyses in this study were performed using SPSS ver. 23 (IBM Corp., Armonk, NY, USA). Comparison analysis between categorical variables was conducted using chi-square test or Fisher exact test. Univariate and multivariate survival analyses were carried out using the Kaplan-Meier method with log-rank test and Cox proportional hazards regression model. All p-values were considered to indicate statistically significant differences if less than 0.05.

RESULTS

Variable amounts of *F. nucleatum* according to tumor location bowel subsite in CRCs

Among the 593 stage II/III CRCs treated with oxaliplatin-based adjuvant chemotherapy (FOLFOX or CAPOX), intratumoral *F. nucleatum* DNA was detected in 408 cases (68.8%). Each *F. nucleatum*-positive CRC was classified as *F. nucleatum*–high or –low based on *F. nucleatum* DNA load, using a cut-off median value of $2^{-\Delta Ct}$. The proportions of *F. nucleatum*–high, –low, and –negative CRCs along the tumor location bowel subsite varied

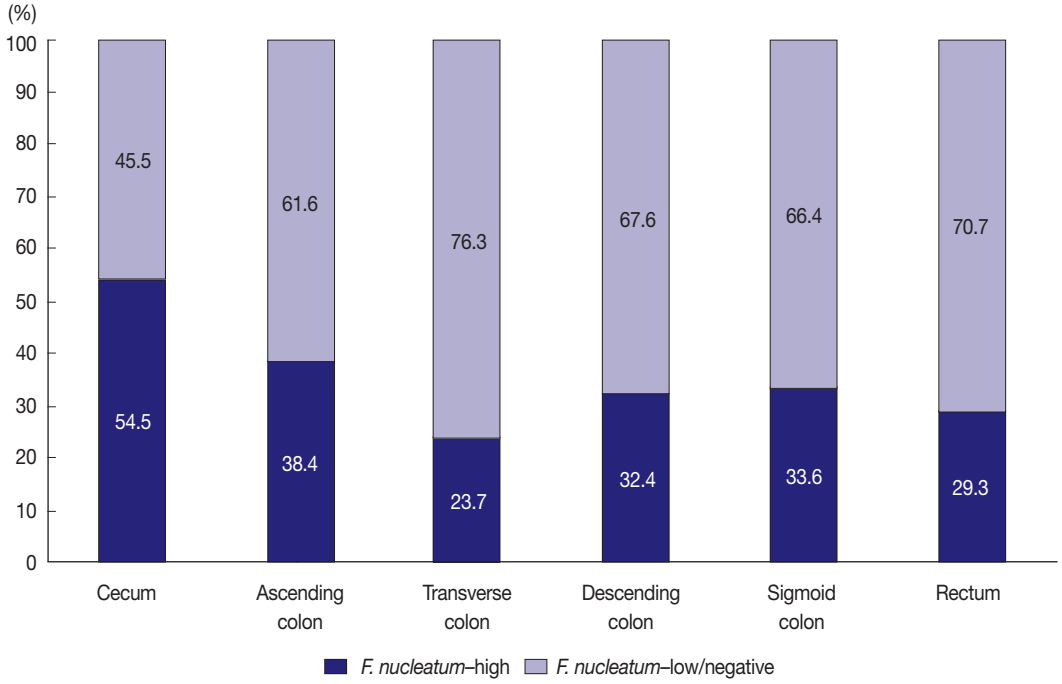

Fig. 1. Different proportions of *Fusobacterium nucleatum*–high vs *F. nucleatum*–low/negative colorectal cancers according to tumor location bowel subsites.

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(Fig. 1). The proportion of *F. nucleatum*–high tumors was highest among cecal cancers, whereas that of *F. nucleatum*–high tumors was lowest among transverse colon cancers (54.5% and 23.7%, respectively) (Fig. 1).

Clinicopathological and molecular associations of *F. nucleatum* in CRCs

We analyzed the relationship between *F. nucleatum* status (high vs. low/negative) and clinicopathological (age, sex, tumor sidedness, pT/pN categories, tumor grade, lymphovascular and perineural invasions, and mucinous histology) and molecular characteristics (MSI, CIMP, and *KRAS*/*BRAF* mutations) in overall stage II/III CRCs treated with oxaliplatin-based adjuvant chemotherapy (n = 593). The results are summarized in Table 1. Among the variables, the pT category was the only factor with statistical significance. *F. nucleatum*–high was significantly associated with advanced pT stage (pT3/pT4) (p = .005) (Table 1). CIMP-high and *KRAS* mutations were more frequent in *F. nucleatum*–high CRCs than in *F. nucleatum*–low/negative CRCs, without statistical significance (p = .174 and p = .093, respectively) (Table 1).

Prognostic impact of *F. nucleatum* in CRCs treated with adjuvant chemotherapy

In survival analysis, no significant difference in disease-free survival (DFS) was evident between the *F. nucleatum* high and *F. nucleatum* low/negative groups in overall 593 stage II/III CRC patients treated with oxaliplatin-based adjuvant chemotherapy (log-rank p = .671) (Fig. 2A). In addition, the prognostic significance of *F. nucleatum* was not identified in subgroups stratified by MSI status (log-rank p = .858 in MSI-high CRCs (n = 40), log-rank p = .625 in MSS/MSI-low CRCs (n = 545) (Supplementary Fig. S2). However, subgroup analyses according to tumor location demonstrated that DFS of the *F. nucleatum*–high group was significantly better than that of the *F. nucleatum*–low/negative group in patients with adjuvant FOLFOX or CAPOX-treated colon cancer located in the non-sigmoid colon (from cecum to descending colon, n = 219) (log-rank p = .026) (Fig. 2B). In sigmoid colon and rectal cancer patients treated with oxaliplatin-based adjuvant chemotherapy (n = 374), the *F. nucleatum*–high group showed a tendency toward worse DFS compared to the *F. nucleatum*–low/negative group, but this survival difference was not statistically significant (log-rank p = .199) (Fig. 2C). In multivariate analysis, *F. nucleatum*–high was an independently favorable prognostic factor in non-sigmoid colon cancer patients treated with oxaliplatin-based adjuvant chemotherapy (hazard ratio, 0.42; 95% confidence interval, 0.18 to 0.97; p = .043) (Table 2).

Table 1. Characteristics of adjuvant chemotherapy-treated, stage II/III CRCs according to the *Fusobacterium nucleatum* status

Variable	F. nucleatum–high	F. nucleatum–low/negative	p-value
Age			.286
Younger (<59 yr)	84 (41.2)	178 (45.8)	
Older (≥59 yr)	120 (58.8)	211 (54.2)	
Sex			.925
Male	124 (60.8)	238 (61.2)	
Female	80 (39.2)	151 (38.8)	
Tumor sidedness			.287
Right-sided	69 (33.8)	115 (29.6)	
Left-sided	135 (66.2)	274 (70.4)	
Gross tumor type			.243
Polypoid/fungating	119 (58.3)	246 (63.2)	
Ulceroinfiltrative	85 (41.7)	143 (36.8)	
pT category			.005
pT1/ pT2	9 (4.4)	44 (11.3)	
pT3/pT4	195 (95.6)	345 (88.7)	
pN category			.464
pN0	34 (16.7)	56 (14.4)	
pN1/pN2	170 (83.3)	333 (85.6)	
Tumor histological grade			.687
G1/G2	188 (92.2)	362 (93.1)	
G3/G4	16 (7.8)	27 (6.9)	
Lymphovascular invasion			.419
Absent	112 (54.9)	200 (51.4)	
Present	92 (45.1)	189 (48.6)	
Perineural invasion			.171
Absent	143 (70.1)	293 (75.3)	
Present	61 (29.9)	96 (24.7)	
Mucinous histology			.269
Absent	184 (90.2)	361 (92.8)	
Present	20 (9.8)	28 (7.2)	
MSI status[a]			.647
MSS/ MSI-low	185 (92.5)	360 (93.5)	
MSI-high	15 (7.5)	25 (6.5)	
CIMP status[b]			.174
CIMP-low/negative	189 (92.6)	369 (95.3)	
CIMP-high	15 (7.4)	18 (4.7)	
KRAS mutation[c]			.093
Absent	137 (67.2)	286 (73.7)	
Present	67 (32.8)	102 (26.3)	
BRAF mutation			.213
Absent	200 (98)	374 (96.1)	
Present	4 (2)	15 (3.9)	

Values are presented as number (%).
CRC, colorectal cancer; G1, grade 1 (well differentiated); G2, grade 2 (moderately differentiated); G3, grade 3 (poorly differentiated); G4, grade 4 (undifferentiated); MSI, microsatellite instability; MSS, microsatellite-stable; CIMP, CpG island methylator phenotype.
[a]Among the 593 cases, MSI status could not be determined in eight cases due to inadequate DNA quality or quantity; [b]Among the 593 cases, CIMP status could not be determined in two cases due to inadequate DNA quality or quantity; [c]Among the 593 cases, *KRAS* mutation could not be determined in one case due to inadequate DNA quality or quantity.

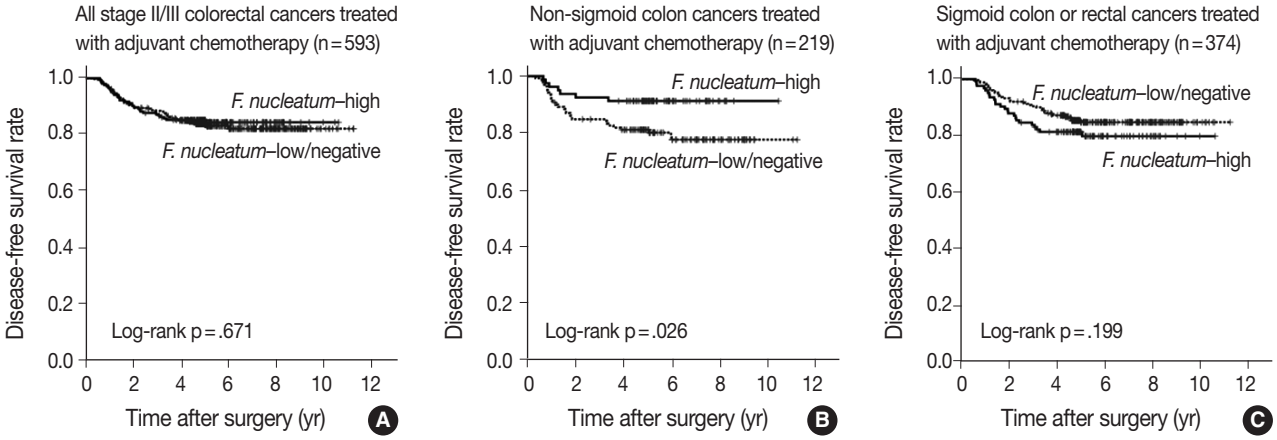

Fig. 2 . Kaplan-Meier survival analysis, including subgroup analysis according to tumor location. (A) No significant difference in disease-free survival was evident between *Fusobacterium nucleatum*–high and –low/negative subgroups in the overall 593 stage II/III colorectal cancer patients treated with oxaliplatin-based adjuvant chemotherapy. (B) The *F. nucleatum*–high subgroup was significantly associated with better disease-free survival in non-sigmoid colon cancer patients treated with oxaliplatin-based adjuvant chemotherapy (n=219). (C) In sigmoid colon and rectal cancer patients treated with oxaliplatin-based adjuvant chemotherapy (n=374), the *F. nucleatum*–high subgroup shows a tendency toward worse disease-free survival without statistical significance.

To further identify the molecular basis of the favorable prognostic effect of *F. nucleatum* observed in non-sigmoid colon cancers, we analyzed the prognostic impact of *F. nucleatum* in subsets of non-sigmoid colon cancer patients according to MSI status. In an MSS/MSI-low subset of non-sigmoid colon cancer patients treated with adjuvant chemotherapy (n = 185), DFS was significantly better in the *F. nucleatum*-high group than in the *F. nucleatum*-low/negative group (log-rank p = .014) (Fig. 3A). However, significant DFS difference according to *F. nucleatum* status was not observed in an MSI-high subset of non-sigmoid colon cancer patients (n = 31) (log-rank p = .844) (Fig. 3B). Finally, survival analyses in MSS/MSI-low (n = 360) and MSI-high (n = 9) subgroups of sigmoid colon or rectal cancers treated with oxaliplatin-based adjuvant chemotherapy demonstrated tendencies toward worse DFS of *F. nucleatum*–high group than of *F. nucleatum*–low/negative group, but there was no statistical significance (log-rank p = .193 in MSS/MSI-low subgroup, Fig. 3C; log-rank p = .885 in MSI-high subgroup, Fig. 3D)

DISCUSSION

Direct or indirect roles of gut microbiota in the pathogenesis of a variety of human diseases have been recently proposed. The demonstration of the close association between *F. nucleatum* and CRC has prompted exploration of the pathogenetic, prognostic, and predictive roles of *F. nucleatum* in CRC. However, there are still limited data regarding the prognostic and predictive values of *F. nucleatum* in CRC. Several studies using clinical samples have indicated that intratumoral *F. nucleatum* is potentially associated with poor prognosis in CRC patients.[3,11,20] Moreover, an experimental study suggested that *F. nucleatum* might be able to induce resistance to chemotherapy by modulating autophagy in CRC cells.[4] Based on the emerging prognostic significance and potential predictive value of *F. nucleatum* in CRC, we decided to investigate the prognostic relevance of *F. nucleatum* in CRCs treated with adjuvant chemotherapy. Most patients with stage III or high-risk stage II CRC are treated with adjuvant chemotherapy after curative surgery to prevent tumor recurrence. Thus, we collected a large series of stage III or high-risk stage II CRCs treated with oxaliplatin-based adjuvant chemotherapy. The survival differences in patient subgroups according to DNA amount of intratumoral *F. nucleatum* measured by qPCR were statistically analyzed. We found that a high load of intratumoral *F. nucleatum* was independently correlated with improved survival in patients with stage II/III non-sigmoid colon cancer treated with oxaliplatin-based adjuvant chemotherapy (Table 2).

There is a discrepancy between our research and previous studies. Several previous studies revealed that *F. nucleatum*–high CRC patients group tended to have shorter disease-specific survival than *F. nucleatum*–low/negative CRC patients group.[3,11,20] However, in the current study, *F. nucleatum* had different prognostic impacts based on tumor location in CRCs treated with adjuvant chemotherapy. In detail, tumors with high levels of *F. nucleatum* had better prognosis than those with low or negative levels of *F. nucleatum* in non-sigmoid colon cancers, including cecum, ascending colon, transverse colon, and descending colon

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Table 2. Univariate and multivariate survival analyses of patients with stage II/III non-sigmoid colon cancer treated with oxaliplatin-based adjuvant chemotherapy (n=219)

Variable	No.	Univariate analysis HR (95% CI)	p-value	Multivariate analysis HR (95% CI)	p-value
F. nucleatum					
F. nucleatum–low/negative	139	Reference		Reference	
F. nucleatum–high	80	0.4 (0.18–0.92)	.031	0.42 (0.18–0.97)	.043
pT category					
pT1/pT2/pT3	183	Reference		Reference	
pT4	36	5.13 (2.65–9.92)	<.001	5.04 (2.53–10.07)	<.001
pN category					
pN0/pN1	176	Reference		Reference	
pN2	43	2.88 (1.47–5.64)	.002	2.65 (1.31–5.35)	.007
Lymphovascular invasion					
Absent	129	Reference		Reference	
Present	90	2.78 (1.41–5.50)	.003	1.39 (0.66–2.95)	.387
Perineural invasion					
Absent	169	Reference		Reference	
Present	50	2.81 (1.45–5.45)	.002	2.92 (1.41–6.05)	.004
BRAF mutation					
Absent	204	Reference		Reference	
Present	15	3.12 (1.30–7.49)	.011	2.21 (0.86–5.69)	.1
Tumor histological grade					
G1/G2	190	Reference		-	
G3/G4	29	1.14 (0.44–2.92)	.791	-	-
MSI status[a]					
MSS/MSI-low	185	Reference		-	
MSI-high	31	0.57 (0.17–1.87)	.353	-	-
CIMP status[b]					
CIMP-low/negative	192	Reference		-	
CIMP-high	25	1.32 (0.51-3.40)	.567	-	-
KRAS mutation[c]					
Absent	148	Reference		-	
Present	71	0.93 (0.46-1.89)	.844	-	-

HR, hazard ratio; 95% CI, 95% confidence interval of HR; G1, grade 1 (well differentiated); G2, grade 2 (moderately differentiated); G3, grade 3 (poorly differentiated); G4, grade 4 (undifferentiated); MSI, microsatellite instability; MSS, microsatellite-stable; CIMP, CpG island methylator phenotype.
[a]Among the 219 cases, MSI status could not be determined in three cases due to inadequate DNA quality or quantity; [b]Among the 219 cases, CIMP status could not be determined in two cases due to inadequate DNA quality or quantity.

cancers (Table 2, Fig. 2B). On the other hand, *F. nucleatum*–high CRCs showed a tendency toward worse prognosis compared to *F. nucleatum*–low/negative CRCs in sigmoid colon and rectal cancers (Fig. 2C). Since these contrasting prognostic implications of *F. nucleatum* according to tumor location may counterbalance the overall prognostic effect of *F. nucleatum* in CRCs, presently *F. nucleatum* displayed no association with prognosis in a total of 593 stage II/III CRC patients treated with adjuvant chemotherapy (Fig. 2A). The reason for the discrepancy between the current and prior findings may be the difference in the composition of the study populations. Yamaoka *et al*.[20] described that *F. nucleatum* was highly correlated with shorter disease specific survival especially in stage IV CRCs. In that study, in all stages of CRCs, disease-specific survival was decreased in CRCs featuring a high level of *F. nucleatum* compared with that in CRCs with low levels of *F. nucleatum*, although the survival differences according to *F. nucleatum* level was decreased compared to that in the stage IV CRC subgroup.[20] In addition, it cannot be excluded that there might be heterogeneities of detailed treatment approaches, such as adjuvant chemotherapy regimen, in the CRC cohorts of other studies. By contrast, our study samples were a well-selected and relatively-homogeneous cohort that contained only stage III or high-risk stage II CRCs treated with oxaliplatin-based adjuvant chemotherapy. Therefore, the prognostic implications of *F. nucleatum* in CRC that is evident from our study could be meaningfully different from the results of other research groups.

In an experimental study, *F. nucleatum* promoted resistance to chemotherapy in CRC cells.[4] However, our results indicate that

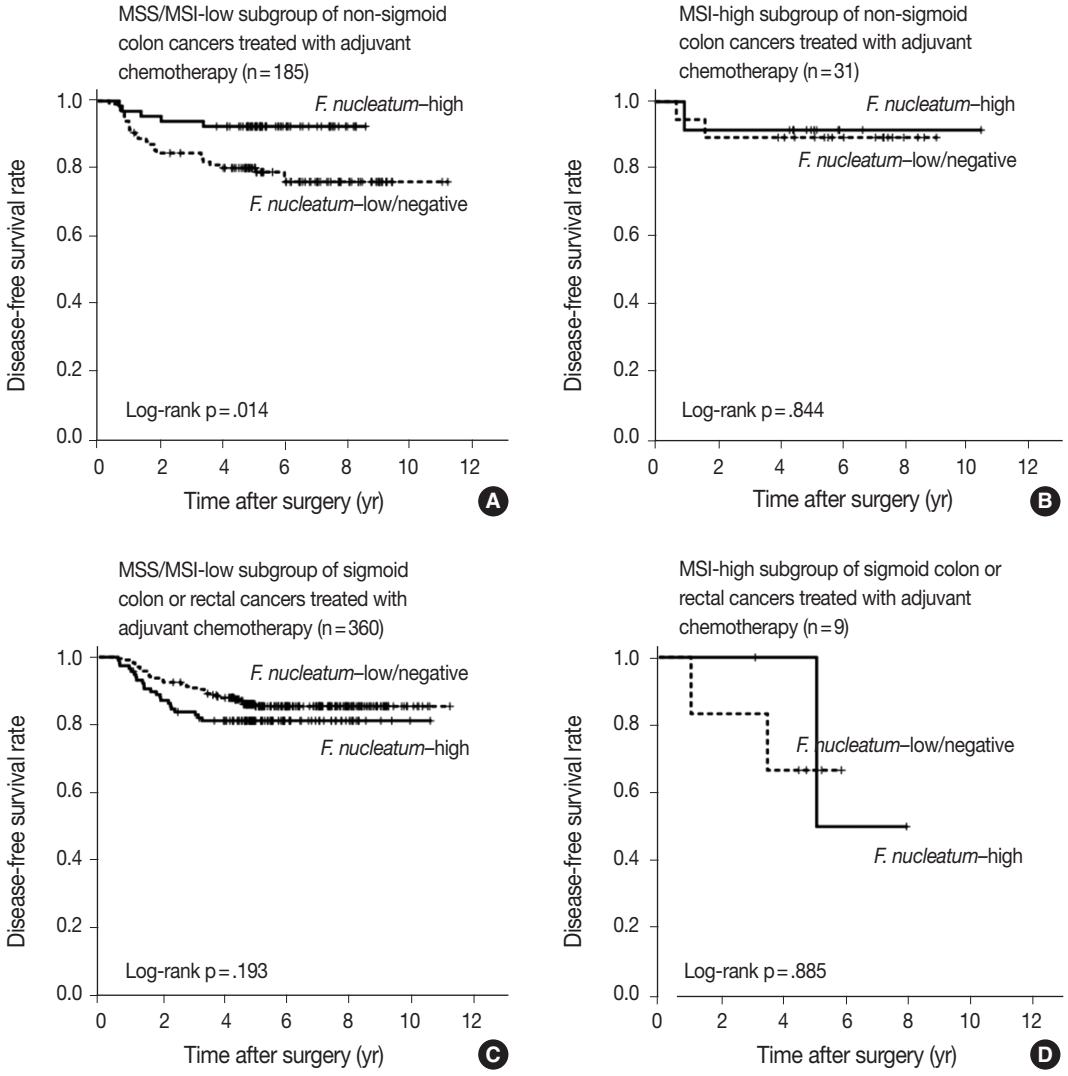

Fig. 3. Kaplan-Meier survival analysis, including subgroup analysis according to combined tumor location and microsatellite instability (MSI) status. (A) The *Fusobacterium nucleatum*–high subgroup was significantly associated with better disease-free survival in an MSS/MSI-low subset of non-sigmoid colon cancer patients treated with oxaliplatin-based adjuvant chemotherapy (n=185). (B) No significant survival difference according to *F. nucleatum* status was observed in an MSI-high subset of non-sigmoid colon cancer patients treated with oxaliplatin-based adjuvant chemotherapy (n=31). (C) There is a tendency toward worse survival in the *F. nucleatum*–high subgroup without statistical significance in an MSS/MSI-low subset of sigmoid colon or rectal cancer patients treated with oxaliplatin-based adjuvant chemotherapy (n=360). (D) No significant survival difference according to *F. nucleatum* status was observed in an MSI-high subset of sigmoid colon or rectal cancer patients treated with oxaliplatin-based adjuvant chemotherapy (n=9).

influences of *F. nucleatum* on responses to chemotherapy might be diverse in the context of tumor location of CRCs. In sigmoid colon and rectal cancers, the expected chemoresistant effect of *F. nucleatum* seems to have occurred because *F. nucleatum*–high was linked with poor prognosis in sigmoid colon and rectal cancer patients treated with adjuvant chemotherapy, although statistical significance was not reached (Fig. 2C). Nevertheless, in non-sigmoid colon cancers, a chemoresistant role of *F. nucleatum* seems to be attenuated. Rather, *F. nucleatum* might induce a chemoresponsive effect because *F. nucleatum*–high was significantly associated with favorable prognosis in non-sigmoid colon cancers treated with adjuvant chemotherapy (Table 2, Fig. 2B). The underlying mechanism of the potential contrasting effects of *F. nucleatum* on the chemotherapy response depending on location of CRC is unclear. However, the idea that different tumor locations can define different prognosis and treatment responses in CRC has been increasingly addressed. In fact, based on the accumulating clinical data, primary tumor location is regarded as a prognostic factor in metastatic CRCs.[21] Stage IV CRCs primarily located in the right-sided colon are significantly associated with worse prognosis

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compared with left-sided stage IV CRCs. The different molecular, pathological, and clinical features between right-sided colon cancers and left-sided CRCs have been reported.[21,22] Therefore, the potential different impacts of *F. nucleatum* on prognosis and treatment responses according to tumor location in CRCs are not surprising. To the best of our knowledge, this study is the first report to investigate the prognostic effect of *F. nucleatum* according to tumor location in CRCs, especially in adjuvant chemotherapy-treated CRCs. Our study suggests that the prognostic effect of *F. nucleatum* should be evaluated considering the location of the tumor.

In this study, the proportion of *F. nucleatum*–high CRCs differed in each tumor location bowel subsite. The proportion of *F. nucleatum*–high tumors in all the CRCs was 34.4% (204 of 593). Cecal cancers displayed the highest proportion of *F. nucleatum*-high tumors (54.5%), followed by ascending colon cancers (38.4%) (Fig. 1). It was notable that over half of the cecal cancers were *F. nucleatum*–high tumors. Our results are consistent with those of previous studies demonstrating the significant association of the proximal location of CRCs with a high level of intratumoral *F. nucleatum*. According to the study by Mima *et al*.,[13] the proportion of *F. nucleatum*–high CRCs increased along the distance from the anal verge, and cecal cancers showed the highest proportion of *F. nucleatum*–high subtype. The underlying mechanism of the specific enrichment of *F. nucleatum* in cecal and ascending colon cancers is still unclear, but microenvironmental or biological factors specifically found in the cecal to ascending colon areas could influence the increase of intratumoral *F. nucleatum*. For example, bacterial biofilms are intensively enriched in right-sided colon tumors compared with those in left-sided colorectal tumors.[23] Based on recent experimental findings, potential molecular mechanisms can be hypothesized. According to a previous experimental study, *F. nucleatum* is enriched in colorectal tumor tissue by Fap2 binding to Gal-GalNAc expressed on tumor cells.[24] Thus, it can be hypothesized that Gal-GalNAc expression on tumor cells might be more upregulated in the right-sided colon than in the left-sided colon. Further investigations are needed to elucidate the biological reason of the preference of invasive *F. nucleatum* for right-sided colon cancers.

According to the recent data reported by Ogino group, *F. nucleatum* in CRCs differentially impacts tumor-infiltrating lymphocyte (TIL) density depending on MSI status.[25] In detail, there was an inverse association between *F. nucleatum* load and TIL density in MSI-high CRCs, whereas a positive correlation between *F. nucleatum* load and TIL density was observed in non–MSI-high CRCs.[25] This finding can provide an important clue for the interpretation of our present results. It has been validated that high TIL density is strongly associated with favorable prognosis in CRCs.[26] Thus, because *F. nucleatum*–high tumors might be associated with increased antitumor immunity and subsequent improved prognosis in non–MSI-high CRCs, the favorable prognostic effect of *F. nucleatum*–high in the MSS/MSI-low subset of non-sigmoid colon cancers, which was observed in our present study, could be a reasonable finding. However, we also found that the prognostic significance of *F. nucleatum* was valid only in non-sigmoid colon cancers, but not in sigmoid colon/rectal cancers, suggesting that both tumor location and MSI status should be concurrently considered for understanding the prognostic implications of *F. nucleatum* in CRCs.

There have been several reports regarding the poor prognostic effect of *F. nucleatum* in CRCs, which was mainly observed in Western CRC cohorts or stage IV CRC cohorts.[3,20,27] However, our present data indicate that high intratumoral *F. nucleatum* load might be associated with favorable prognosis in a limited subgroup of CRCs, a MSS/MSI-low subset of non-sigmoid colon cancers. We suspect that different compositions of tumor locations and MSI subtypes in CRC cohorts might influence the different prognostic effects of *F. nucleatum* in overall CRCs. Because it has been known that the frequency of MSI-high in CRCs is definitely lower in East Asia countries than in Western countries,[28] the potential favorable prognostic effect of *F. nucleatum* in proximal colonic-located, non–MSI-high CRCs might be significantly attenuated in CRC cohorts of Western countries, which consist of relatively high numbers of MSI-high tumors. Instead, both the tendency toward worse prognosis of *F. nucleatum*–high in MSI-high tumors (Supplementary Fig. S2A) and the potential poor prognostic effect of *F. nucleatum*–high tumors observed in sigmoid colon/rectal cancers (Fig. 2C) might augment the adverse prognostic impact of *F. nucleatum* in overall CRCs. To confirm this hypothesis, additional investigations using various CRC cohorts having different ethnic backgrounds would be needed. Regarding the poor prognostic feature of *F. nucleatum* in stage IV CRCs observed in a few studies,[20,27] it could be explained by relatively high proportion of distal-located CRCs as primary origin of stage IV CRCs. Thus, the potential worse prognostic effect of *F. nucleatum* in sigmoid colon or rectal cancers might be augmented especially in a stage IV subset of CRCs.

Although significant associations between CIMP-high (and/or MSI-high) and *F. nucleatum* in CRCs were reported in several previous studies,[3,8,9] significant correlation between *F. nucleatum*–high group and CIMP-high or MSI-high molecular subtype was not observed in our present study (Table 1). However, there was an evident tendency toward higher proportion of

CIMP-high tumors in *F. nucleatum*-high group than in *F. nucleatum*–low/negative group (7.4% vs. 4.7%) (Table 1). In addition, we performed mean comparison of *F. nucleatum* DNA amount ($2^{-\Delta Ct}$) between CIMP-high and CIMP-low/negative tumors, and the results indicated that mean *F. nucleatum* DNA amount was higher in CIMP-high tumors than in CIMP-low/negative tumors although statistical significance was not reached (0.986 vs. 0.367, p = .157) (Supplementary Fig. S3). The reason for unclear molecular association of *F. nucleatum* in our study samples may be explained by potential ethnic differences and biased sample composition. As mentioned above, the frequencies of MSI-high and CIMP-high in CRCs are lower in East Asian population than in Western population. If a high number of CIMP-high cases were included in our cohort, significant association between *F. nucleatum*–high and CIMP-high might have been observed. Moreover, our study samples were confined to selected stage III or high-risk stage II CRCs treated with adjuvant chemotherapy. Thus, molecular compositions of our CRC cohort were possibly biased. For example, the CIMP-high/non-MSI-high subtype has been known as an aggressive phenotype of CRCs and can be more enriched in stage IV tumors. Because stage IV cases were excluded from our study samples, the potential association between *F. nucleatum*–high and CIMP-high could be weakened. Considering that data are limited, the relationship between *F. nucleatum* and specific molecular phenotypes in CRCs has not been conclusive yet. Therefore, further clinical and experimental investigations are needed to elucidate whether CIMP-high and/or MSI-high molecular phenotype can significantly interact with intratumoral *F. nucleatum* enrichment in CRCs.

The proportion of *F. nucleatum*-positive cases in CRCs by qPCR analysis has been variable according to different investigations (8.6%–74%).[29] In our results, *F. nucleatum* DNA was detected in 408 out of 593 cases (68.8%). The reason for variability in the *F. nucleatum*-positive rate in CRCs is unclear, but tissue quality might be a critical factor for this discrepancy. Recently, Lee *et al*.[27] found that the tissue fixation method could affect different results of *F. nucleatum* qPCR analysis. We also found that when the FFPE tissues were more recent, the positive rate of *F. nucleatum* was increased (unpublished data). Therefore, it can be inferred that *F. nucleatum*-positive rate by qPCR method could be variable, depending on tissue fixation method and tissue storage time.

There are several limitations in this study. First, we assessed the amount of *F. nucleatum* in genomic DNA samples extracted from FFPE tissues. The precise quantification of *F. nucleatum* could be disturbed owing to the degraded nature of DNA extracted from FFPE tissues although a substantial number of previous studies that analyzed *F. nucleatum* in clinical CRC samples also used FFPE tissue-derived DNA. Second, our study cohort was retrospectively collected. The results from our study should be validated by other prospective studies.

In conclusion, the prognostic impact of *F. nucleatum* in CRCs treated with adjuvant chemotherapy may differ depending on the combined status of primary tumor location and MSI molecular phenotype. Intratumoral *F. nucleatum* load may be a potential prognostic factor in stage III or high-risk stage II non-sigmoid colon cancers treated with oxaliplatin-based adjuvant chemotherapy, especially in an MSS/MSI-low molecular subtype. There have been very limited data regarding the detailed prognostic implications of *F. nucleatum* in CRCs according to various clinicopathologic and molecular contexts. Therefore, further studies using large prospective cohorts will be necessary to validate the different location/MSI-dependent prognostic impacts of *F. nucleatum* in CRCs treated with adjuvant chemotherapy.

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Electronic Supplementary Material

Supplementary materials are available at Journal of Pathology and Translational Medicine (http://jpatholtm.org).

Conflicts of Interest

The authors declare that they have no potential conflicts of interest.

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