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ORIGINAL RESEARCH

Clinical Significance of *Fusobacterium nucleatum* and Microsatellite Instability in Evaluating Colorectal Cancer Prognosis

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Objective: Both genetic and microbial factors play important roles in colorectal cancer (CRC) development. The effects of *Fusobacterium nucleatum* (*F. nucleatum*) and microsatellite instability (MSI) on CRC prognosis require more clinical evidence. We aimed to investigate the role of *F. nucleatum* and MSI as biomarkers in predicting the prognosis of CRC.

Methods: CRC patients in various TNM stages were enrolled. MSI status and *F. nucleatum* were detected by immunohistochemical staining of formalin-fixed paraffin-embedded (FFPE) specimens. The associations between MSI status and *F. nucleatum* and clinical parameters were analyzed.

Results: MSI tumors were more frequently observed in the colon than in the rectum. Cancerous tissues had higher levels of *F. nucleatum* than adjacent noncancerous tissues. There were no significant differences in *F. nucleatum* abundance in different age, sex, tumor stage, location, and tumor marker groups. MSI status was associated with tumor location and stage. Survival analyses revealed that disease-free survival (DFS) was significantly longer in the *F. nucleatum*-negative, younger age, and TNM stage I–II groups (p < 0.05), and age, advanced TNM stage (III and IV), and *F. nucleatum* status were independent factors for poor prognosis. Multivariate Cox regression and receiver operating characteristic (ROC) curve analyses showed that conventional tumor biomarkers of CRC had more prognostic value than *F. nucleatum* and MSI.

Conclusion: Age, advanced TNM stage, and *F. nucleatum* positivity were independent factors of poor prognosis, suggesting that *F. nucleatum* and MSI may contribute to the identification of new strategies for the prevention and treatment of CRC.

Keywords: colorectal cancer, microsatellite instability, Fusobacterium nucleatum, prognostic, factors

Introduction

The incidence of CRC has increased significantly in the last 10 years. Currently, CRC ranks second in terms of the number of cancer-related deaths in men and women.¹ Although surgery and subsequent chemotherapy have made significant progress in CRC treatment, the mortality of CRC remains very high, with 25% of CRC patients displaying metastasis at diagnosis and approximately 50% of those treated eventually developing metastasis during their lifetime. Furthermore, the five-year survival rate for metastatic CRC is low, remaining at approximately 14%.² Thus, the existing achievements appear to be insufficient to improve the clinical outcome of CRC patients, and therefore, substantial efforts are still underway to identify other potential CRC-related driving factors.³

The carcinogenesis of CRC is a complicated multistep process that involves intrinsic and extrinsic factors. Extrinsic factors, including inflammation from hyperactivated immune cells, the release of proinflammatory cytokines, or gut dysbiosis, can lead to an inflammatory and possibly premalignant environment. Intrinsic factors include the accumulation

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of mutations in tumor suppressor genes and oncogenes, as well as sporadic mutations that lead to mutation-induced CRC (sporadic CRC).⁴ CRC is mainly associated with at least three distinct genetic pathways: MSI, chromosomal instability (CIN), and CpG island methylator phenotype (CIMP).⁵ MSI represents one of the major types of genomic instability in human cancers and is the most common in CRC. DNA mismatch repair (MMR) is responsible for proofreading replication errors in microsatellites and involves four proteins (MLH1, MSH2, PMS2, and MSH6) interacting with each other as heterodimer complexes.⁶ Most hereditary nonpolyposis CRCs and 15% of sporadic CRCs are characterized by MSI.⁷ CRC patients with deficient MMR (dMMR) respond well to standard chemotherapy regimens.⁸ Recently, immune checkpoint inhibitor therapy (ICI) was found to be effective in a specific subset of CRC patients with dMMR and high microsatellite instability (MSI-H) and ineffective in patients with proficient mismatch repair (pMMR) and low microsatellite instability (MSI-L).⁹ Clinical observation showed that MLH1/MSH2-negative patients have a more favorable overall survival than MLH1/MSH2-positive patients. Thus, MLH1/MSH2 could be an independent prognostic and predictive factor for the outcome of stage II–III CRC.¹⁰

CRC carcinogenesis may result from dysbiosis in the colonic microbiota, with an increased proportion of certain bacteria whose metabolism produces cytotoxic or genotoxic compounds that cause DNA damage either through the production of free radicals or through the abnormal activation of resident immune cells.¹¹ It is now emerging that specific bacteria are implicated in the risk of CRC.¹² *F. nucleatum* has been found to be an initial trigger in CRC development and elicits a proinflammatory microenvironment around the tumor to drive tumor formation and progression.^{13,14} Studies have demonstrated enrichment of *F. nucleatum* in human colorectal adenomas and carcinomas compared to adjacent normal tissue.¹⁵ Increased tissue *F. nucleatum* DNA has been associated with advanced disease stage and shorter colorectal cancer-specific survival.¹⁶ Our previous study showed an overabundance of *F. nucleatum* in the fecal samples of CRC patients; however, there was no significant difference in the distribution of *F. nucleatum* in patients with different tumor sites, and it was associated with patient prognosis.¹⁷ Therefore, we investigated whether the expression of this bacterium in the tissue has prognostic significance. Even though novel evidence for *F. nucleatum* associated colorectal carcinogenesis is accumulating, few clinical studies have investigated its role in prognostic evaluation, especially when combined with microsatellite instability in CRC. In addition, there is still a lack of detailed knowledge regarding the biological functions, genetics, pathologic characteristics, and clinical significance of *F. nucleatum* in CRC.¹⁸

Both intrinsic and extrinsic factors play important roles in CRC development and prognosis. *F. nucleatum* has been suggested to be enriched in the MSI-H molecular subtype of CRC,¹⁶ and more *F. nucleatum* in CRC tissue is associated with higher degrees of MSI and the CpG island methylator phenotype (CIMP).¹⁹ However, the clinicopathologic and molecular factors interacting with *F. nucleatum* in MSI-H CRC are poorly understood.²⁰ The prognostic role of *F. nucleatum* may differ among patients with different disease statuses.¹⁸ Evidence indicates that the molecular features of CRC, especially MSI, can influence antitumor T-cell-mediated adaptive immunity,²¹ and the amount of tissue *F. nucleatum* is inversely associated with CD3+ T-cell density in CRC tissue.²² Currently, the antitumoral immune response has shown great success specifically in patients with metastasized MSI cancers.²³ The effects of *F. nucleatum* and MSI on CRC prognosis still require more clinical evidence. Elucidating the role of *F. nucleatum* and MSI in the prognosis of CRC, risk assessment, prognostication, and therapeutic opportunities.²⁴

Methods

The ethics committee of the First Affiliated Hospital of Shantou University Medical College approved this study (approval number: B-2020-209). Written informed consent was obtained from each patient for the use of their archived specimens in medical research. From January 2010 to June 2018, CRC patients who underwent curative surgical resection and had a complete medical history were enrolled in this study. CRC was confirmed by pathological diagnosis. Patients with a history of polyps, adenomas, or nonprimary colorectal cancer were excluded. The formalin-fixed paraffinembedded (FFPE) specimens archived in the First Affiliated Hospital of Shantou University Medical College were analyzed retrospectively. Tumor-node-metastasis (TNM) stage was determined according to the latest guidelines of the Union for International Cancer Control (8th edition).

Immunohistochemical (IHC) Analyses

Tumor tissues and corresponding paracancerous tissues from the same patients were fixed with formalin, embedded in paraffin, and serially sectioned at a thickness of 4 µm. After the wax was dissolved in the oven at 60 °C for 1.5 h, the samples were immediately rehydrated in decreasing concentrations of ethanol (3 times total, 10 min each time). Immunohistochemical staining was performed for the tumor biomarkers HER-2 (rabbit monoclonal antibody, catalog number: RMA-0690), CgA (mouse monoclonal antibody: MAB-0202), Syn (rabbit monoclonal antibody, catalog number: RAB-0155), CD31 (mouse monoclonal antibody, catalog number: MAB-0031), and D2-40 (mouse monoclonal antibody, catalog number: MAB-0567) (Maxim, China). The cancerous and paracancerous tissues were analyzed according to the manufacturers' instructions. In the negative control sections, the primary antibody was omitted.

MSI status was based on four markers (MLH1, MSH2, MSH6, and PMS2). Patients whose tissue exhibited positive staining for all markers were considered microsatellite stable (MSS)/MSI-L, whereas patients whose tissue was negative for the expression of at least one marker were considered MSI-H.²⁵ MMR protein (MLH1, PMS2, MSH2, and MSH6) expression in tumor tissue was detected using manufacturer-recommended automated staining protocols on a BOND-III Fully Automated IHC and ISH Stainer (Leica Microsystems; Melbourne, Australia). The MMR antibodies (MLH1, PMS2, MSH2, and MSH6) used in this study were clones ES05, MOR4G, 25D12, and PU29, respectively (Novocastra; New Castle, UK). Thus, all patients were dichotomized as MSI-negative (microsatellite stable or low instability) or MSI-H (mismatch repair protein-deficient). The standard strain for *F. nucleatum* (ATCC 25586) cultured in vitro was used as a positive control for staining. All patients were divided into two groups, *F. nucleatum*-positive and *F. nucleatum*-negative groups (Figure 1). The IHC staining was evaluated and graded by two pathologists. Patient prognosis was assessed by disease-free survival (DFS) calculated from the date of surgery to the date of local recurrence or regional/ distant metastasis.

Statistical Analysis

Statistical analyses were performed using SPSS (Version 20.0). All continuous variables are reported as medians with interquartile ranges (IQRs). Spearman rank correlation was used to test the associations among parameters. Multivariate regression analysis by Cox regression was applied to determine independent factors affecting CRC prognosis. The Kruskal–Wallis test was used to compare clinicopathologic variables and stage-matched CRC outcomes. Associations between clinicopathologic factors and disease-free survival in CRC patients were initially assessed by univariable Cox proportional hazards regression. Multivariate Cox regression analysis assessing the relationships with *F. nucleatum* levels initially included sex (male vs female), age (continuous), tumor location (proximal colon vs distal colon and rectum), MSI status (MSI vs MSS), tumor markers, and consideration of potential confounding and causal relationships. Survival curves were calculated using the Kaplan–Meier method, and survival comparisons were made using Mantel's Log rank test. In multivariate analyses, the Cox proportional hazards regression model was used to estimate hazard ratios and 95% confidence intervals (CIs) to determine independent risk factors associated with DFS. Principal component analysis was performed to obtain significant principal components. Receiver operating characteristic (ROC) curve analysis was performed to determine the usefulness of the biomarkers for discriminating between CRC and others. SPSS for Windows version 20.0 was used for statistical analyses (SPSS Incorporated, Chicago, IL, USA). Values of p<0.05 were considered statistically significant.

Results

In total, 184 CRC patients were included in this study. The male:female ratio was 1.22:1. Eighty-five CRC patients were less than 60 years old, while 99 patients were older than 60 years (mean age: 60.89 years, range: 21–83 years). CRC patients were grouped based on TNM stage; 116 patients were stage I–stage II, and 68 patients were stage III–stage IV. The tumor locations were the proximal colon (47 patients), distal colon (62 patients), and rectum (75 patients). IHC staining showed that 103 tumor tissue samples were positive for HER-2, 22 samples were positive for CgA, 26 samples were positive for Syn, 147 samples were positive for CD31, and 147 samples were positive for D2-40. The clinico-pathological data of the CRC patients are summarized in Table 1.



Figure I (A) Gram stain: Gram-negative bacteria appear red when stained. Original magnification 1000×. (B) *F. nucleatum* was positive by immunohistochemistry, original magnification 1000×. C-P: Immunohistochemical detection. Original magnification 400×. (C) Negative *F. nucleatum* expression in paracancerous tissue, (D) positive *F. nucleatum* expression in paracancerous tissue, (E) negative *F. nucleatum* expression in cancer tissue, (F) positive *F. nucleatum* expression in cancer tissue, (G) negative MLH-1 expression, (H) positive MLH-1 expression, (I) negative MSH2 expression, (J) positive MSH2 expression, (K) negative PMS2 expression, (L) positive PMS2 expression, (M) negative MSH6 expression, (O) negative HER-2 expression, (P) positive HER-2 expression.

Clinical and Laboratory Biomarkers in MSI and MSS CRC Patients

We assessed the MSI status of the FFPE tissue specimens of CRC patients. There were 22 (12%) patients who had MSI and 162 (88%) patients who had MSS. Different groups of patients with MSI did not differ in terms of age or sex. MSI tumors were significantly more frequent in the colon than in the rectum (ratio: 4.5:1). Of the 22 patients with MSI, none were in TNM stage I, 11 (50%) were in TNM stage II, 7 (32%) were in TNM stage III, and 4 (18%) were in TNM stage IV (Table 2).

F. nucleatum Levels in CRC Tissue and Adjacent Noncancerous Tissue

Analysis of *F. nucleatum* in patient tumor tissue indicated that cancerous tissues had higher *F. nucleatum* than adjacent noncancerous tissues, with positive rates of 38.1% in cancerous tissues and 23.7% in adjacent noncancerous tissues.

Factors	No. of the Patients	Percentage,%		
Sex				
Male	101	54.9%		
Female	83	45.1%		
Age (years)				
<60	85	46.2%		
≥60	99	53.8%		
Tumor location				
Proximal colon	47	25.5%		
Distal colon	62	33.7%		
Rectum	75	40.8%		
TNM Stages				
Stage I	21	11.4%		
Stage II	95	51.6%		
Stage III	26	14.1%		
Stage IV	42	22. 9 %		
HER-2				
Negative	68	39.8%		
Positive +	67	39.2%		
Positive ++	36	21.0%		
CD31				
Negative	25	14.5%		
positive	147	85.5%		
D2-40				
Negative	25	14.5%		
Positive	147	85.5%		
Syn				
Negative	155	85.6%		
Positive	26	14.4%		
CgA				
Negative	159	87.8%		
Positive	22	12.2%		

Table I Clinical Features of CRC Patients

Abbreviations: CRC, colorectal cancer; HER-2, human epidermal growth factor receptor-2; Syn, synaptophysin; CgA, chromogranin A; TNM, Tumor-node-metastasis stage was determined according to the latest guidelines of the Union for International Cancer Control (8th edition).

Weak *F. nucleatum* antigen expression was observed in 60 cases of cancerous tissue and 74 cases of noncancerous tissue, while strongly positive *F. nucleatum* antigen expression was observed in 37 cases of cancerous tissue and 23 cases of noncancerous tissue (p<0.001) (Table 3). Among 37 positive samples, 5 cases (13.5%) were TNM stage I, 13 cases (35.1%) were TNM stage II, 5 cases (13.5%) were TNM stage III, and 14 cases (37.9%) were TNM stage IV. Among 23 cases of *F. nucleatum* antigen-positive noncancerous tissue samples, 3 cases (13.0%) were from TNM stage I patients, 9 cases (39.1%) were from TNM stage II patients, 3 cases (13.0%) were from TNM stage II patients, and 8 cases (34.9%)

Table	2 Clinical	and Molecular	Features o	f CRC	According t	to Microsatelli	te Status n	(%)	
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Factors(n)	MSI (n=22)	MSS (n=162)	χ ²	df	P value
Sex			1.783	1	0.182
Male(101) Female(83)	15 (14.9) 7 (8.4)	86 (85.1) 76 (91.6)			
Age			0.701	I	0.402
<60 (85) ≥60 (99)	12 (14.1) 10 (10.1)	73 (85.9) 89 (89.9)			
Tumor location			11.659	2	0.003**
Proximal colon(47) Distal colon(62) Rectum(75)	12 (25.5) 6 (9.7) 4 (5.3)	35 (74.5) 56 (90.3) 71 (94.7)			
TNM Stages			8.633	3	0.035*
Stage I (21) Stage II(95) Stage III (26) Stage IV(42)	0 (0) 11 (11.6) 7 (26.9) 4 (9.5)	21 (100) 84 (88.4) 19 (73.1) 38 (90.5)			
HER-2			6.248	2	0.044*
Negative (68) Positive +(67) Positive ++(36)	14 (20.6) 6 (9.0) 2 (5.6)	54 (79.4) 61 (91.0) 34 (94.4)			
CD31			14.126	1	<0.01**
Negative (25) Positive (147)	9 (36.0) 13 (8.8)	16 (64.0) 134 (91.2)			
D2-40			14.126	1	<0.01**
Negative (25) Positive (147)	9 (36.0) 13 (8.8)	16 (64.0) 134 (91.2)			
Syn			0.297	I	0.586
Negative(155) Positive (26)	18 (11.6) 4 (15.4)	137 (88.4) 22 (84.6)			
CgA			1.778	1	0.182
Negative(159) Positive (22)	22 (13.0) 0 (0)	147 (87.0) 22 (100)			

Note: p < 0.05 and p < 0.01 were considered to be statistically significant.

Abbreviations: df, degree of freedom; CRC, colorectal cancer; HER-2, human epidermal growth factor receptor-2; Syn, synaptophysin; CgA, chromogranin A; MSS, microsatellite stability; MSI, microsatellite instability.

were from TNM stage IV patients. However, there was no significant difference in *F. nucleatum* levels among the different TMN stages (p>0.05) (Table 4).

Correlations Among F. nucleatum, MSI, and Other Clinicopathological Features of Patients with CRC

Spearman rank correlation analysis was performed to evaluate the possible interactions among *F. nucleatum*, MSI status, and other biomarkers reflecting the characteristics of CRC patients. There were no significant differences in *F. nucleatum*

Table 3 Comparison of the Positive Rate of F. nucleatum Infection in CRC Tissues and Adjacent Noncancerous Ti	ssues in Patients with
CRC n (%)	

		Carcinoma Tissues			df	P value
		F. nucleatum (-) 60(61.9)	F. nucleatum(+) 37(38.1)			
adjacent noncancerous tissues	F. nucleatum (-) 74(76.3)	60(81.1)	14(18.9)	48.89	I	<0.01**
	F. nucleatum (+) 23(23.7)	0(0)	23(100)			

Note: ** p<0.01 were considered to be statistically significant.

Abbreviations: df, degree of freedom; CRC, colorectal cancer; F. nucleatum, Fusobacterium nucleatum.

abundance between different age, sex, tumor stage, location, and tumor marker groups (Table 5). In addition, the expression of *F. nucleatum* was not associated with MSI status, age, or tumor biomarkers. MSI status was associated with HER-2 positivity (r=-0.21, p=0.039) and tumor location (r=-0.254, p=0.013), and TNM stage was associated with HER-2 positivity (r=-0.196, p=0.036), CD31 positivity (r=-0.252, p=0.007), D2-40 (r=-0.252, p=0.007), and tumor location (r=-0.25, p=0.007) (Table 6).

Prognostic Value of F. nucleatum and MSI

To further estimate the value of *F. nucleatum* and MSI in predicting the risk of CRC, multivariate Cox regression and receiver operating characteristic (ROC) curve analyses were performed. The marker with the highest sensitivity was age (88.2%), followed by TNM stage (73.5%), while the marker with the highest specificity was CgA (95.2%), followed by Syn (81.0%). *F. nucleatum* had a sensitivity and specificity of 50.5% and 68.3%, respectively, and MSI had a sensitivity and specificity of 26.5% and 79.4%, respectively. These results indicate that the conventional biomarkers of CRC had more diagnostic value than *F. nucleatum* and MSI (Table 7).

Cox regression analysis (univariate and multivariate) of the model included all biomarkers studied, and after screening step by step, age (HR 0.125, 95% CI 0.041–0.379, p< 0.001), TNM stages (HR 0.270, 95% CI 0.114–0.637, p=0.003), *F. nucleatum* (HR 0.423, 95% CI 0.183–0.974, p=0.043) and tumor location (HR 2.626, 95% CI 1.006–6.852, p=0.049) had statistical significance. Confounding factors were included, and variables significantly associated (p<0.05) with CRC in univariate analysis, including age, tumor location, TNM stage, and *F. nucleatum* infection, were entered into multivariate Cox regression analysis. The results showed that age, advanced TNM stage (stage III and IV), and *F. nucleatum* positivity were independent predictors of poor prognosis (Table 8).

Further investigation was performed using Kaplan–Meier survival curves to evaluate the suitability of *F. nucleatum*, MSI, and other tumor biomarkers as prognostic factors. The median follow-up was 52 weeks (range: 1–345 weeks). The mean levels of the parameters were considered for the stratification of variables for survival time analysis. The results showed that the DFS (disease-free survival) (weeks) in the *F. nucleatum*-negative group was significantly longer than that in the *F. nucleatum*-positive group (87.77±10.466 vs 67.19±11.940, p=0.041). Older people (>60 years old) had a significantly shorter DFS than younger people (<60 years old) (108.21±15.605 vs 61.69±7.547, p<0.001), and patients

	Carcinoma Tissues		df	P value	Adjacent Nonc	df	P value	
	F. nucleatum (-)	F. nucleatum (+)			F. nucleatum(-)	F. nucleatum (+)		
TNM stages			3	0.452			3	0.633
Stage I	13	5			15	3		
Stage II	17	13			21	9		
Stage III	13	5			15	3		
Stage IV	17	14			23	8		

 Table 4 F. nucleatum Infection in CRC Tissues and Adjacent Noncancerous Tissues in CRC Patients with Different TNM

 Stages

Abbreviations: df, degree of freedom; CRC, colorectal cancer; F. nucleatum, Fusobacterium nucleatum.

Factors (n = 97) F. nucleatum		um	df	X ²	P value	
	Negative(n=60)	Positive(n=37)				
Sex			I	1.773	0.183	
Male(58)	39(67.2)	19(32.8)				
Female(39)	21(53.8)	18(46.2)				
Age			I	0.047	0.829	
<60 (38)	23(60.5)	15(39.5)				
≥60 (59)	37(62.7)	22(37.3)				
Tumor location			2	0.614	0.736	
Proximal colon(27)	18(66.7)	9(33.3)				
Distal colon(30)	17(56.7)	13(43.3)				
Rectum(40)	25(62.5)	15(37.5)				
TNM stages			3	2.629	0.452	
Stage I(48)	13(72.2)	5(27.8)				
Stage II(48)	17(56.7)	13(43.3)				
Stage III(48)	13(72.2)	5(27.8)				
Stage IV(49)	17(54.8)	14(45.2)				
HER-2			I	0.029	0.866	
Negative(43)	27(62.8)	16(37.2)				
Positive(54)	33(61.1)	21(38.9)				
CD31			I	1.387	0.239	
Negative (25)	3(52.0)	12(48.0)				
Positive(72)	47(65.3)	25(34.7)				
D2-40			I	1.387	0.239	
Negative(25)	13(52.0)	12(48.0)				
Positive(72)	47(65.3)	25(34.7)				
Syn			1	1.403	0.236	
Negative(81)	48(59.3)	33(40.7)				
Positive(16)	12(75.0)	4(25.0)				
CgA			Į.	0.736	0.391	
Negative(92)	56(60.9)	36(39.1)				
Positive(5)	4(80.0)	l (20.0)				

Table 5 Clinical and Molecular Features of CRC According to F. nucleatum Infection

Abbreviations: df, degree of freedom; CRC, colorectal cancer; *F. nucleatum, Fusobacterium nucleatum*; HER-2, human epidermal growth factor receptor-2;Syn, synaptophysin;CgA, chromogranin A.

with TNM stage III–IV had a significantly shorter DFS than those with TNM stage I–II (64.02 ± 8.338 vs 96.15 ± 13.295 , p=0.001). Patients with colon cancer had a shorter DFS than those with rectal cancer (64.07 ± 8.409 vs 102.50 ± 14.476 , p=0.004). Patients with MSS tended to have a shorter DFS than patients with MSI (78.16 ± 9.088 vs 85.91 ± 16.637 , p=0.829), and patients with negative HER-2, CD31 D2-40, and Syn expression tended to have a shorter DFS than patients with positive expression, but the differences did not reach statistical significance (p>0.05) (Table 9). Kaplan–Meier curves illustrate unadjusted differences in CRC (Figure 2) across subtypes. The observed patterns of survival differences were maintained in multivariable-adjusted analyses.

	Sex	Age	F. nucleatum	MSI	HER-2	CD31	D2-40	TNM stages
Age	r=0.098 p=0.336							
F. nucleatum	r=0.087 p=0.396	r= -0.049 p=0.630						
MSI	r= 0.093 p=0.364	r= -0.120 p=0.239	r=-0.013 p=0.902					
HER-2	r=0.182 p=0.075	r=0.049 p=0.631	r=-0.039 p=0.701	r=-0.21* p=0.039				
CD31	r=0.051 p=0.620	r=0.107 p=0.297	r=-0.004 p=0.969	r= -0.187 p=0.066	r=0.138 p=0.175			
D2-40	r=0.051 p=0.620	r=0.107 p=0.297	r=-0.004 p=0.969	r= -0.187 p=0.066	r=0.138 p=0.175	r=1.00 p<0.001**		
TNM stages	r=0.001 p=0.991	r= -0.005 p=0.960	r=-0.026 p=0.781	r= -0.018 p=0.851	r= -0.196 p=0.036*	r=-0.252 p=0.007**	r=-0.252 p=0.007**	
Tumor Location	r= 0.046 p=0.651	r= -0.186 p=0.069	r=-0.073 p=0.474	r= -0.254 p=0.013*	r=0.115 p=0.259	r=0.158 p=0.121	r=0.158 p=0.121	r=-0.250 p=0.007**

Table 6 Correlation Analysis of Clinical-Pathological Indices with C	RC
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Notes: Age is divided into two levels: \geq 60 and <60. *p <0.05 and **p<0.01 were considered to be statistically significant. **Abbreviations**: CRC, colorectal cancer; *F. nucleatum, Fusobacterium nucleatum*; HER-2, human epidermal growth factor receptor-2; MSI, microsatellite instability.

Table	7 Receiver	Operating	Characteristic	Curve Anal	ysis of Parameters
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Variable	AUC	Std. Error	95% CI	P value	Sensitivity(%)	Specificity (%)
Age	0.711	0.053	0.607–0.815	0.001**	88.2	54.0
TNM stages	0.677	0.057	0.566–0.789	0.004**	73.5	61.9
F. nucleatum	0.591	0.061	0.471-0.711	0.139	50.0	68.3
Sex	0.530	0.062	0.409-0.651	0.626	44. I	61.9
MSI	0.529	0.062	0.407–0.651	0.637	26.5	79.4
CgA	0.506	0.506	0.384–0.582	0.928	5.9	95.2
Syn	0.464	0.061	0.35–0.582	0.555	11.8	81.0
CD31	0.449	0.062	0.327–0.571	0.412	67.6	22.2
D2-40	0.449	0.062	0.327–0.571	0.412	67.6	22.2
HER-2	0.411	0.061	0.292-0.531	0.150	44. I	38.1
Tumor location	0.364	0.058	0.250-0.477	0.027*	23.5	49.2

Notes: AUC indicates the ability of the biomarkers and combined components to analyze prognosis; *p <0.05 and **p<0.01 were considered to be statistically significant.

Abbreviations: AUC area under the curve; Std, standard; 95% Cl, 95% confidence interval; *F. nucleatum, Fusobacterium nucleatum*; HER-2, human epidermal growth factor receptor-2; MSI, microsatellite instability; Syn, synaptophysin; CgA, chromogranin A.

Table 8 Cox Regression Analysis of Prognostic	c Factors in Patients with CRC
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Clinical Variables	В	SE	Wald	df	HR	95% CI	P value
	Univariate cox analysis						
Sex(male vs female)	-0.293	0.347	0.711	I	0.746	0.378-1.473	0.399
Age(≥60 vs <60)	1.889	0.534	12.513	1	0.151	0.053–0.431	<0.001**
Tumor location (Colon vs Rectum)	1.128	0.411	7.546	1	3.089	1.382-6.909	0.006**
TNM stages(Stage III-IV vs Stage I-II)	1.213	0.393	9.526	1	3.364	1.557–7.268	0.002**
MSI status(MSI vs MSS)	0.084	0.391	0.046	1	1.088	0.505–2.342	0.830
F. nucleatum (Positive vs Negative)	0.690	0.345	4.0	1	1.994	1.014-3.922	0.045*
HER-2(Positive vs Negative)	0.575	0.346	2.758	1	0.563	0.286-1.109	0.097
CD31(Positive vs Negative)	0.382	0.370	11.064	1	1.465	0.7709–3.627	0.302
D2-40(Positive vs Negative)	0.382	0.370	11.064	1	1.465	0.7709–3.627	0.302
CgA(Positive vs Negative)	0.256	0.732	0.122	1	0.774	0.184-3.251	0.122
Syn(Positive vs Negative)	0.470	0.533	0.777	T	1.600	00.563-4.548	0.378
	Multivariate Cox analysis						
Sex(male vs Female)	0.105	0.418	0.063	I	1.110	0.490–2517	0.802
Age(≥60 vs <60)	2.080	0.567	13.472	1	0.125	0.041-0.379	<0.001**
Tumor location (Colon vs Rectum)	0.965	0.489	3.891	1	2.626	1.006-6.852	0.049*
TNM stages(Stage III-IV vs Stage I-II)	1.311	0.438	8.939	1	0.270	0.114-0.637	0.003**
MSI status(MSI vs MSS)	0.507	0.454	1.246	I	0.602	0.247–1.467	0.264
F. nucleatum (Positive vs Negative)	0.861	0.426	4.085	I	0.423	0.183–0.974	0.043*

Notes: *p <0.05 and **p<0.01 were considered to be statistically significant.

Abbreviations: B, beta; SE, standard error; df, degree of freedom; HR, hazard ratio;95% Cl, 95% confidence interval; CRC, colorectal cancer; *F. nucleatum, Fusobacterium nucleatum*; HER-2, human epidermal growth factor receptor-2; MSI, microsatellite instability; MSS, microsatellite stability; Syn, synaptophysin; CgA, chromogranin A.

Factors	DFS(Weeks±SD)	df	P value
Sex		I	0.395
Male Female	(86.14±10.864) (70.67±11.368)		
Age		I	<0.001**
<60 ≥60	(61.69±7.547) (108.21±15.605)		
Tumor location		I	0.004*
Colon Rectum	(64.07±8.409) (102.50±14.476)		
TNM stages		1	0.001**
Stage I-II Stage III-IV	(96.15±13.295) (64.02±8.338)		
MSI status		I	0.829
MSS MSI	(78.16±9.088) (85.91±16.637)		

Table 9 Univariate Analysis of the Impact of Possible Risk Factors

 on Disease-Free Survival in CRC Patients

(Continued)

Factors	DFS(Weeks±SD)	df	P value
F. nucleatum		I	0.041*
Negative Positive	(87.77±10.466) (67.19±11.940)		
HER-2		I	0.091
Negative Positive	(70.47±10.831) (87.91±16.637)		
CD31		I	0.297
Negative Positive	(70.52±13.744) (83.18±9.599)		
D2-40		I	0.297
Negative Positive	(70.52±13.744) (83.18±9.599)		
CgA		I	0.371
Negative Positive	(81.21±8.325) (56.20±14.263)		
Syn		I	0.724
Negative Positive	(78.8±8.693) (85.56±20.106)		

Table 9 (Continued).

Note: *p <0.05 and **p<0.01 were considered to be statistically significant. **Abbreviations:** DFS, disease-free survival; df, degree of freedom; CRC, colorectal cancer; *F. nucleatum, Fusobacterium nucleatum*; HER-2, human epidermal growth factor receptor-2; MSI, microsatellite instability; MSS, microsatellite stability; Syn, synaptophysin; CgA, chromogranin A.

Principal Component Analysis (PCA) of Candidate Parameters

The aim of principal component analysis (PCA) is to reveal data that best explain the variance of the parameters. To identify the profile that provides greater class prediction accuracy than a single biomarker, we used PCA to select a panel with the greatest accuracy of class prediction and the smallest misclassification error. PCA extracted four important principal components with eigenvalues >1, explaining 68.55% of the total variance in the dataset. The first model showed positive loadings (>0.50) for age, MSI, HER-2, and CD31. The second model showed strong positive loadings (>0.50) for CgA and Syn. The third model included sex and HER-2, while the fourth model included tumor location and *F. nucleatum* (Table 10). Then, the ROC curves of the components were used to determine the value of the four components in CRC diagnosis (Table 11). The sensitivity and specificity of components 1, 2, 3, and 4 were not higher than those of the single parameters.

Discussion

It is estimated that genetic factors account for only 10–30% of the CRC risk, while environmental factors may play a significant role in causing sporadic CRC.^{26,27} The interaction between genetic background and environmental stimuli contributes to the development of CRC.²⁸ Thus, these two factors should be considered simultaneously when evaluating the risk factors for CRC prognosis. Presently, MSI is used extensively in hospitals as a parameter for guiding treatment. Unfortunately, the microbiota has rarely been measured and considered as a parameter in clinical practice.



Figure 2 Kaplan–Meier survival curves were used to evaluate the suitability of these molecules as prognostic factors. The results showed that the DFS in the *F. nucleatum*-negative group was significantly longer than that in the *F. nucleatum*-positive group. Older people (>60 years old) had a significantly shorter DFS than younger people (<60 years old), and patients with TNM stage III–IV had significantly a shorter DFS than those with TNM stage I–II. Patients with colon cancer had a shorter DFS than those with rectal cancer.

The gut microbiota is associated with chemotherapy and immunotherapy responses in solid tumors.²⁹ *F. nucleatum* has been identified as a crucial pathogen in CRC carcinogenesis, promoting tumor progression and inhibiting antitumor immune responses in the colorectum.^{30,31} In addition, *F. nucleatum* induces resistance of CRC cells to oxaliplatin and 5-FU by selectively decreasing miR18a and miR-4802, then activating the autophagy pathway and preventing cells from posttreatment apoptosis, and finally promoting cancer recurrence.³² The close association between *F. nucleatum* and CRC

Variable	Component I	Component 2	Component 3	Component 4
Sex	0.383	0.034	0.797	0.116
Age	0.644	0.154	-0.195	-0.025
Tumor location	0.166	0.384	-0.081	-0.571
TNM stages	0.434	0.324	0.512	-0.327
MSI	- 0.825	-0.127	0.098	-0.195
F. nucleatum	-0.012	-0.029	0.091	0.760
HER-2	0.572	0.108	0.599	-0.058
CD31	0.670	0.207	-0.346	-0.114
CgA	-0.012	0.908	-0.060	0.246
Syn	-0.43 I	0.767	-0.060	0.267

Table 10 Loading Scores of Variables on the First Three Significant Principal Components ThatCombined Components for the Analysis of Prognosis

Notes: Bold values indicate strong and moderate loadings: Component I has strong positive loadings on Age, HER-2, CD31 and negative loadings on MSI; Component 2 has positive loadings on CgA, Syn; Component 3 has positive loadings on Sex,TNM stages and HER-2; Component 4 has positive loadings on *F. nucleatum* and negative loadings on Tumor location. **Abbreviations**: CRC, colorectal cancer; *F. nucleatum, Fusobacterium nucleatum*; HER-2, human epidermal growth factor receptor-2; MSI, microsatellite instability; Syn, synaptophysin; CgA, chromogranin A.

Variable	AUC	Std. Error	95% CI	P value	Sensitivity(%)	Specificity(%)
Component I	0.537	0.063	0.414-0.660	0.550	61.8	57.1
Component 2	0.613	0.057	0.500-0.725	0.068	76.5	50.8
Component 3	0.523	0.068	0.390-0.655	0.711	58.8	20.6
Component 4	0.415	0.065	0.288-0.541	0.166	58.8	14.3

 Table II Receiver Operating Characteristic Curve Analysis of Components

Notes: AUC indicates the ability of the biomarkers and combined components to analyze prognosis.

Abbreviations: AUC, area under the curve; Std, standard; 95% Cl, 95% confidence interval.

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.³³ Cohort studies in multiple countries have confirmed that CRC patients have an increased abundance of F. nucleatum in feces,³⁴ and CRC patients with a higher fecal abundance of F. nucleatum had a 3- to 5-fold increased risk of being diagnosed with rectal cancer compared to colon cancer, specifically right-sided colon cancer.³⁵ However, feces are easily affected by diet, bowel habits, and other factors,³⁶ and the abundance of bacteria is greatly affected by the sampling process. Considering that microbiota in direct contact with epithelial cells are likely to influence the progression of CRC, microbiome analysis of tissue samples can provide highly useful data.³⁷ F. nucleatum can actively promote colorectal tumorigenesis.³⁰ There is a significant correlation between intratumoral F. nucleatum positivity and nuclear β-catenin accumulation in human CRC tissues.³⁸ Fecal samples cannot truly reflect the luminal microbiota corresponding to tumor tissues, which is a great limitation to studying their direct pathogenic effects on CRC.^{37,39} Our results show that cancerous tissues have higher levels of F. nucleatum than adjacent noncancerous tissues, consistent with previous results that F. nucleatum is a specifically enriched species within the tumor tissue of human CRC.^{16,31,32} F. nucleatum positivity tended to vary depending on tumor stage, with higher positive rates of intratumoral F. nucleatum being observed in patients with TNM stage IV disease. However, F. nucleatum may increase according to the histological grade and may play a role in the early stage of colorectal carcinogenesis.¹⁵ We also analyzed the relationship between F. nucleatum levels (high vs low/ negative) and clinicopathological features but did not find marked associations between F. nucleatum status and other clinicopathological features. The abundance of F. nucleatum in the gut is very low compared to that of other gut bacteria, and its role may be limited to the local area of the gut rather than the whole body. Another reason may be the inhomogeneity of F. nucleatum distribution, consistent with a previous study showing that the abundance of F. nucleatum varies at different sampling sites even in the same tumor tissues⁴⁰ and tumor site.³⁵ A high load of intratumoral F. nucleatum is associated with a poor response to chemotherapy.^{16,32} In this study, Kaplan–Meier analysis showed that F. nucleatum in CRC is associated with patient DFS, and the DFS in the F. nucleatum-negative group was significantly longer than that in the F. nucleatum-positive group. Consistent with previous studies, our results show that intratumoral F. nucleatum is potentially associated with poor prognosis in CRC patients.^{16,41}

Previous studies also show that a high load of intratumoral *F. nucleatum* is associated with MSI,^{15,16,32} suggesting that *F. nucleatum* may play an active role in the tumorigenesis of MSI-H CRC.^{30,31} MMR genes are highly conserved housekeeping genes. MMR maintains correct DNA replication and high fidelity by repairing DNA base mismatches, which allows genomic stability and reduces spontaneous mutations.⁴² MSI is characterized by the loss of DNA methylation or MMR caused by a genetic mutation, which leads to widespread alterations in the length of short repeated sequences.⁴³ Deficiency in MMR genes, usually hMSH2 or hMLH1, promotes CRC development due to mutation or silencing.⁴⁴ We found that patients with MSS tended to have a shorter DFS than patients with MSI. Previous studies revealed that MSI status is associated with survival.⁴⁵ However, Gkekasetal pooled 19 studies containing 5998 cases and found no significant association of MSI with OS or disease-free survival (DFS) for stage II CRC.⁴⁶ Our correlation analysis suggested that MSI-H status was associated with tumor location but was not associated with *F. nucleatum*. The prognostic significance of *F. nucleatum* was also not identified in subgroups stratified by MSI status.³³

Given the high mortality and poor treatment outcomes in advanced stages of CRC, developing more effective treatment options is an urgent need. Currently, probiotic therapy represents an emerging strategy for CRC patients. Microbial and molecular biomarker tests hold promise for personalized therapy. However, a considerable proportion of them may be overestimated, and they are not currently recommended for prognosis prediction or therapy selection due to insufficient evidence.⁴⁷ A recent study revealed that tumor site and MSI status may be crucial characteristics that have prognostic value for tumors treated with adjuvant chemotherapy. However, there are still limited data regarding the prognostic and predictive values of *F. nucleatum* in CRC.³³ Given the heterogeneity of inpatient sample collection/types, therapies administered, and CRC patient populations (eg, by stage or tumor site), extensive studies are needed to understand the diagnostic, therapeutic, and prognostic significance of *F. nucleatum* across the cancer spectrum for patients diagnosed with CRC.³⁵ Several tumor markers (ie, CA19-9, CEA, CgA, and Syn) might also reflect CRC prognosis, but their specificity and sensitivity remain unclear. Using a combination of tumor markers and microbiota improves the sensitivity of diagnosing CRC compared to using tumor markers alone.⁴⁸ Elucidating the role of *F. nucleatum* and MSI may contribute to identifying new strategies for the prevention and treatment of CRC,²⁰ but further evaluation will require a larger sample size to build a simple platform that could rapidly and accurately predict CRC development.

Ethical Approval

The First Affiliated Hospital of Shantou University Medical College Institutional Ethics Board approved the study (approval number: B-2020-209), including all procedures (participant recruitment and all experimental protocols). Studies involving humans conform to the guiding principles of the Declaration of Helsinki. Patients who were selected from the First Affiliated Hospital of Shantou University provided written informed consent.

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Author Contributions

YX, QXZ, YXX and XYJ designed and drafted the manuscript. XYJ revised the final manuscript. MZ collected the samples, ZQF finished the experiment, XL and YMY summarized the clinical data, and YXX and MZ processed the statistical data. YX had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors contributed to the data analysis, drafted or revised the article, agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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Disclosure

The authors report no competing interests in this work.

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