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ORIGINAL RESEARCH

Clinical significance of *Fusobacterium nucleatum*, epithelial–mesenchymal transition, and cancer stem cell markers in stage III/IV colorectal cancer patients

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Abstract: Colorectal cancer (CRC) is a common digestive malignancy and emerging studies have closely linked its initiation and development with gut microbiota changes. *Fusobacterium nucleatum* (*Fn*) has been recently identified as a pathogenic bacteria for CRC; however, its prognostic significance for patients is poorly investigated and is less for patients within late stage. Therefore, in this study, we made efforts to analyze its level and prognostic significance in a retrospective cohort of 280 stage III/IV CRC patients. We found that the *Fn* level was abnormally high in tumor tissues and correlated with tumor invasion, lymph node metastasis status, and distant metastasis. We also identified it as an independent adverse prognostic factor for cancer-specific survival (CSS) and disease-free survival (DFS). The following subgroup analysis indicated that *Fn* level could stratify CSS and DFS in stage IIIB/C and IV patients but failed in stage IIIA patients. In addition, stage III/IV patients with low *Fn* level were found to benefit more from adjuvant chemotherapy than those with high *Fn* level, in terms of DFS. Finally, we analyzed the expression and clinical significance of epithelial-to-mesenchymal transition (EMT) markers (E-cadherin and N-cadherin) and cancer stem cell (CSC) markers (Nanog, Oct-4, and Sox-2) in CRC tissues. The results indicated that N-cadherin, Nanog, Oct-4, and Sox-2 were adverse prognostic factors in these patients, while the opposite was true for E-cadherin. More importantly, expression of E-cadherin, N-cadherin, and Nanog was significantly correlated with *Fn* level in tumor tissues, suggesting the potential involvement of *Fn* in EMT-CSC cross talk during CRC progression. Taken together, these findings indicate that *Fn* is a novel predictive biomarker for clinical management in stage III/IV patients, and targeting *Fn* may be an effective adjuvant approach for preventing CRC metastasis and chemotherapy resistance.

Keywords: colorectal cancer, *Fn*, EMT, cancer stem cell, prognosis

Introduction

Colorectal cancer (CRC) is a fatal digestive malignancy that is commonly diagnosed in both males and females worldwide.[1] In USA, it is the third most common form of cancer and will account for an estimated 135,430 newly diagnosed cases and 50,260 CRC-specific deaths in 2017.[2] In China, its incidence has reached ~37.63 per 100,000 in 2015 according to the latest report.[3] The pathogenesis of CRC is a complicated multistep process involving various inherent and environmental factors such as genetic predisposition and unhealthy lifestyles.[4] Although dramatic reduction has been achieved in CRC mortality because of the introduction of screening programs and multidisciplinary treatments, ~60% of CRC patients are still diagnosed with advanced stage with their 5-year survival rate ranging from 14% to 71%.[5] In addition, there are few effective therapeutical approaches and prognostic biomarkers available for metastatic

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CRC currently, frequently leading to inappropriate decision making.[6] Targeted therapy (such as epidermal growth factor receptor antagonists) represents an emerging clinical strategy for these patients; however, primary and acquired therapy resistance limit its actual efficiency.[7] Molecular biomarker tests hold promise for personalized therapy, while a considerable proportion of them may be overestimated and fail to be recommended for prognosis prediction or therapy selection due to insufficient evidence.[8,9] Therefore, it can be concluded that our existing achievements appear to be insufficient to improve the clinical outcome of CRC patients and therefore substantial efforts are still essential to identify other potential CRC-related driving factors.

Recently, increasing studies have suggested that gut microbiota dysbiosis is correlated with tumor initiation and development.[10] Microbiota dysbiosis may contribute to the malignant progression of cancer cells through various mechanisms such as metabolism signals, inflammation induction, and immunosuppression.[11] Furthermore, microbiota is also crucial for the therapeutical efficacy of some anticancer drugs such as cyclophosphamide, which may associate with its regulation of T-cell responses.[12] In gastrointestinal malignancies, a close correlation between microbiota and carcinogenesis has been well established in gastric cancer, where *Helicobacter pylori* is most extensively studied and has been identified as a risk factor for screening.[13] However, with regard to CRC, related studies are emerging although advanced metagenomic techniques are able to provide more potential pathogenic microbiota.[14] For example, Tsoi et al proved that *Peptostreptococcus anaerobius* is increased in CRC tissues and promotes the growth of CRC cells through inducing intracellular cholesterol synthesis.[15] Wang et al demonstrated that *Enterococcus faecalis* can drive the malignant transformation in normal colon epithelial cells via its bystander effect.[16] Despite increasing evidences supporting the oncogenic role of some specific bacteria in CRC, their clinical significance is still poorly investigated and whether these bacteria can be further developed as clinical biomarkers for patient management remains unknown.

Previously, using pyrosequencing, we found that *Fusobacterium nucleatum* (*Fn*) is abnormally abundant in 1,2-dimethylhydrazine-induced CRC animal models as compared with healthy controls.[17] Then, we used the same method to further confirm that it is also significantly more abundant in human CRC tissues than in adjacent normal tissues, suggesting its potential correlation with CRC development.[18] Further investigation revealed that *Fn* promotes the proliferation and invasiveness of CRC cells through activating toll-like receptors/MyD88/NF-Kb/miR-21 signaling.[19] Given these findings, we speculate that *Fn* may be a promising clinical biomarker for CRC patients. Therefore, in this study, we aimed to investigate the level and clinical significance of *Fn* in stage III/IV CRC patients, who are clinically characterized with positive regional/distant metastasis and have a dramatically worse outcome than those within stage I/II. Since epithelial-to-mesenchymal transition (EMT) and cancer stem cell (CSC) are both widely considered as major molecular factors driving cancer development, we also made efforts to detect the expression of representative EMT and CSC markers in these patients and identify their potential correlations with *Fn*.[20,21] Taken together, our findings not only suggest *Fn* as a novel therapeutical target and prognostic biomarker for CRC patients within late stage, but also highlight the crucial link between dysregulated microbiota and oncogenic molecular events in CRC progression.

Materials and methods

Patient data and specimens

A total of 280 pairs of tumor and adjacent normal tissues were collected from stage III/IV CRC patients who underwent radical surgery at Department of General Surgery, Shanghai Jiao Tong University Affiliated Sixth People's Hospital and Shanghai Tenth People's Hospital between October 1, 2007 and September 25, 2015. All the patients were pathologically confirmed as CRC with positive lymph node metastasis (LNM). Preoperative distant metastasis (including lung, liver, and ovary) was identified by enhanced computed tomography (CT) scanning. Tumor-node-metastasis (TNM) stage was determined according to the latest guidelines of the Union for International Cancer Control (8th edition). Neither preoperative chemotherapy nor radiotherapy was performed on patients. For postoperative chemotherapy, a standard FOLFOX scheme (5-fluorouracil [5-fu] [Shanghai Xudong Haipu Pharmaceutical Co., LTD, Shanghai, China] + oxaliplatin [Jiangsu HengRui Medicine Co., LTD, Lianyungang, Jiangsu, China] + leucovorin [Jiangsu HengRui Medicine Co., LTD, Lianyungang, Jiangsu, China]) was applied. Regular follow-up was conducted according to the Clinical Practice Guidelines in Oncology proposed by the National Comprehensive Cancer Network. In brief, patients were recommended to undergo physical examination, carcinoembryonic antigen (CEA) test, and enhanced CT scan every 3–6 months for the first 2 years, and then 6–12 months for the following 3 years. Patient prognosis was assessed by cancer-specific survival (CSS) and disease-free survival (DFS). CSS was calculated from the date of surgery to the date of death caused by CRC, while DFS was calculated from the date of surgery to the date of local recurrence or regional/

Table 1 Correlations between *Fn* level and clinicopathological parameters in stage III/IV CRC patients

Characteristics	Total	*Fn* level		*p*-value
		Low	High	
Gender				0.705
Female	122	42	80	
Male	158	51	107	
Age				0.822
≤65 years	111	36	75	
>65 years	169	57	112	
Tumor location				0.579
Rectal	130	41	89	
Colon	150	52	98	
Tumor size				0.357
≤5 cm	214	68	146	
>5 cm	66	25	41	
Tumor differentiation				0.650
Poor	74	23	51	
Well/moderate	206	70	136	
Tumor invasion				0.015
T1–T2	96	41	55	
T3–T4	184	52	132	
Lymph node metastasis				0.008
N1	81	17	64	
N2a	95	41	54	
N2b	104	35	69	
Distant metastasis				0.020
Absent	218	80	138	
Present	62	13	49	
Ki-67 expression				0.381
<30%	78	29	49	
≥30%	202	64	138	
Serum CEA level				0.274
≤5 ng/mL	99	37	62	
>5 ng/mL	181	56	125	
BMI				0.202
<18.5 kg/m^2	22	11	11	
18.5–24.99 kg/m^2	178	58	120	
≥25.0 kg/m^2	80	24	56	

Abbreviations: *Fn*, *Fusobacterium nucleatum*; CRC, colorectal cancer; CEA, carcinoembryonic antigen; BMI, body mass index.

distant metastasis. The basic clinical features of patients are summarized in Table 1. This study was approved by the ethics committees of both the hospitals mentioned above. Written informed consents were obtained from patients or their legal guardians for using their specimens in medical researches.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The levels of *Fn* in human CRC and adjacent normal tissues were detected by qRT-PCR. Briefly, paraffin-embedded tissues were deparaffinized in xylene and lysed in buffer ATL (Qiagen NV, Venlo, the Netherlands) and Proteinase K (Qiagen NV). Then, the genomic DNAs were extracted using QIAamp DNA FFPE Tissue Kit according to the manufacturer's instructions (Qiagen NV). The quality of obtained DNAs was verified by an ultraviolet spectrophotometer and eligible DNA samples were preserved at –20°C. The PCR reaction was performed on a 7500 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using SYBR Premix Ex Taq (TaKaRa, Kusatsu, Shiga, Japan). The reaction conditions were applied as follows: initial denaturation at 95°C for 10 minutes, denaturation at 95°C for 1 minute, primer annealing at 60°C for 20 seconds, and primer extension at 56°C for 60 seconds. The sequences of primers were as follows: *Fn*, forward: 5′-CTTAGGAATGAGACAGAGATG-3′ and reverse: 5′-TGATGGTAACATACGAAAGG-3′; β-actin, forward: 5′-CCTCCATCGTCCACCGCAAATG-3′ and reverse: 5′-TGCTGTCACCTTCACCGTTCCA-3′. The $2^{-\Delta\Delta CT}$ method was utilized to calculate the relative level of *Fn* gene and β-actin served as an internal control gene. All the experiments were repeated in triplicate.

Immunohistochemistry (IHC) and staining evaluation

Experimental procedures of IHC were carried out according to our previous study.[22] In brief, paraffin-embedded tissues were continuously cut into 4-μm-thick sections, dewaxed in xylene, and rehydrated in gradient concentrations of ethanol. Antigen retrieval was achieved by microwave heating and endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 solution. Then, sections were incubated with the primary antibody against E-cadherin (1:250; Abcam, Cambridge, UK), N-cadherin (1:250; Abcam), Nanog (1:200; Abcam), Sox-2 (1:200; Abcam), and Oct-4 (1:200; Abcam) at 4°C overnight. Sections incubated with only antibody dilution buffer were utilized as negative controls. Following several washes with phosphate-buffered saline solution, sections were treated with the secondary antibody (1:250; Abcam) at 37°C for 30 minutes. Finally, protein staining was visualized by incubating sections with a diaminobenzidine kit (Thermo Fisher Scientific) for 5 minutes. The sections were counterstained with hematoxylin (Thermo Fisher Scientific) for 10 minutes, dehydrated, sealed, and transferred for microscopic examination.

Staining evaluation was independently carried out by two investigators who were blind to the clinical features and outcome of patients. Any controversial cases were determined by a well-skilled pathologist. The evaluation criteria were based on staining intensity (SI) and percentage of positive cells (PP). SI is scored as follows: 0, negative; 1, weak; 2, moderate; 3, strong. PP is scored as follows: 0, 0%–10%; 1, 11%–25%; 2, 26%–50%; 3, 51%–75%; 4, 76%–100%.

A final staining score was calculated by multiplying the PP score with SI score. The cutoff value of the final score was determined by receiver operating characteristic (ROC) curve analysis. The sections that scored more or less than the cutoff value were regarded as high or low expression cases, respectively.

Statistical analysis

Data are presented as mean ± standard deviation and statistical analyses were performed on SPSS 20.0 statistical software (IBM Corporation, Armonk, NY, USA). The *Fn* level between CRC and adjacent normal tissues was compared by Mann–Whitney test. The cutoff value of the ROC curve was estimated by Youden index. The correlations between biomarkers and clinicopathological parameters were analyzed by chi-square test. The CSS and DFS curves based on Kaplan–Meier model were depicted using GraphPad Prism 5 (GraphPad Software, Inc, La Jolla, CA, USA) and intergroup difference was compared by log-rank test. Independent factors affecting CSS/DFS were identified by univariate and multivariate analysis based on Cox proportional hazards regression model. The impact of *Fn* level on chemotherapy benefits was evaluated using treatment-by-biomarker interaction analysis in a 2×2 factorial design.[23] The correlations of *Fn* level with expression of EMT/CSC markers in CRC tissues were evaluated by Spearman's rank correlation coefficient. A p-value <0.05 was considered statistically significant.

Results

Fn level in CRC and adjacent normal tissues of stage III/IV CRC patients

The relative level of *Fn* in CRC and adjacent normal tissues was detected by qRT-PCR. As shown in Figure 1A,

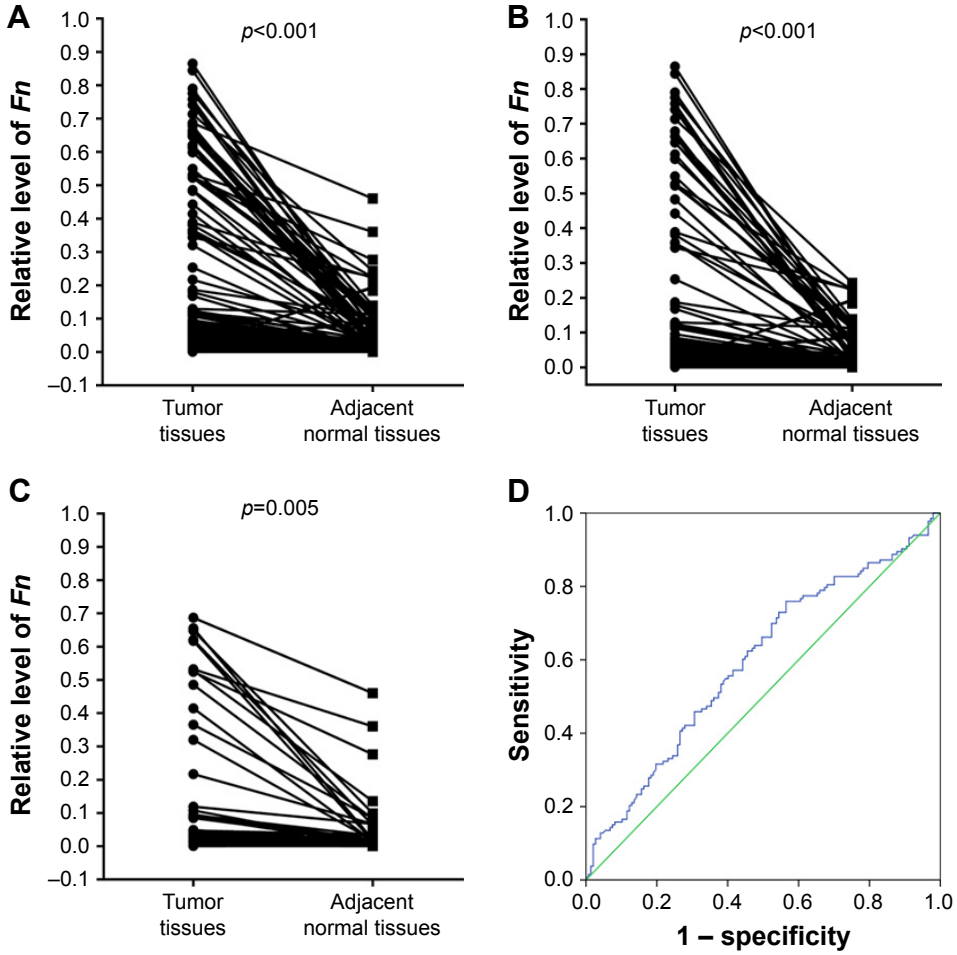

Figure 1 *Fn* level in tumor and adjacent normal tissues of stage III/IV CRC patients.

Notes: (**A**) *Fn* level is significantly higher in tumor tissues than in adjacent normal tissues of the whole cohort (tumor vs normal: 0.1092±0.2150 vs 0.0245±0.0553, n=280, $p<0.001$). (**B**) *Fn* level is significantly higher in tumor tissues than in adjacent normal tissues of stage III patients (tumor vs normal: 0.1043±0.2165 vs 0.0216±0.0450, n=218, $p<0.001$). (**C**) *Fn* level is significantly higher in tumor tissues than in adjacent normal tissues of stage IV patients (tumor vs normal: 0.1266±0.2106 vs 0.0348±0.0817, n=62, $p=0.005$). (**D**) ROC curve analysis determined the cutoff value of *Fn* level in tumor tissues to be 0.0282.

Abbreviations: *Fn*, *Fusobacterium nucleatum*; CRC, colorectal cancer; ROC, receiver operating characteristic.

for the whole study cohort, *Fn* level is significantly higher in CRC tissues than in adjacent normal tissues (CRC vs normal: 0.1092±0.2150 vs 0.0245±0.0553, n=280, $p<0.001$). In subgroups classified by tumor stage, this difference remains statistically significant in both stage III patients (CRC vs normal: 0.1043±0.2165 vs 0.0216±0.0450, n=218, $p<0.001$, Figure 1B) and stage IV patients (CRC vs normal: 0.1266±0.2106 vs 0.0348±0.0817, n=62, $p=0.005$, Figure 1C). Then, the ROC curve was used to calculate an optimal cutoff value for defining the *Fn* level (Figure 1D). The optimal cutoff value of *Fn* level in CRC tissues was 0.0282. Therefore, we classified the entire cohort into a high level group (n=187) and a low level group (n=93) according to this cutoff value.

Correlations between *Fn* level and clinicopathological parameters in stage III/IV CRC patients

As shown in Table 1, *Fn* level was found to significantly associate with tumor invasion ($p=0.015$), LNM status ($p=0.008$), and distant metastasis ($p=0.020$). No significant association was observed between *Fn* level and other clinicopathological parameters including age ($p=0.822$), gender ($p=0.705$), tumor location ($p=0.579$), tumor size ($p=0.357$), tumor differentiation ($p=0.650$), body mass index ($p=0.202$), preoperative serum CEA level ($p=0.274$), and Ki-67 positive rate ($p=0.381$).

Prognostic significance of *Fn* in stage III/IV CRC patients

The impact of *Fn* on patient prognosis was illustrated using Kaplan–Meier survival curves. For the whole cohort, patients with low *Fn* level had a significantly better CSS and DFS than those with high *Fn* level (CSS, $p<0.001$; DFS, $p<0.001$, Figure 2A). As shown in Tables 2 and 3, the univariate analysis suggested that *Fn* level, tumor invasion, LNM status, distant metastasis, and serum CEA level were significant factors for CSS ($p<0.001$, $p=0.015$, $p=0.002$, $p<0.001$, $p=0.046$), while *Fn* level, tumor differentiation, tumor invasion, LNM status, distant metastasis, and serum CEA level were for DFS ($p<0.001$, $p=0.009$, $p=0.005$, $p=0.015$, $p<0.001$, $p=0.018$). The multivariate analysis suggested that *Fn* level, LNM status, distant metastasis, and serum CEA level were independent factors affecting CSS ($p<0.001$, $p=0.001$, $p<0.001$, $p=0.031$), while *Fn* level, tumor differentiation, tumor invasion, LNM status, distant metastasis, and serum CEA level were affecting DFS ($p<0.001$, $p=0.003$, $p=0.022$, $p=0.008$, $p<0.001$, $p=0.027$). To further identify whether *Fn* has the capacity to stratify patient prognosis within the same stage, subgroup analysis was performed according to LNM status and distant metastasis. Surprisingly, we found that stage IIIA patients with low *Fn* level had no better CSS and DFS than those with high *Fn* level (CSS: $p=0.247$; DFS: $p=0.371$, Figure 2B). But, high *Fn* level was still significantly associated with worse CSS and DFS in other stage III patients (stage IIIB: CSS: $p=0.038$, DFS: $p=0.029$, Figure 2C; stage IIIC: CSS: $p=0.035$, DFS: $p=0.048$, Figure 2D). With regard to its prognostic role in stage IV patients, a statistically significant association between high *Fn* level and worse clinical outcome is also obviously found (CSS: $p=0.042$; DFS: $p=0.019$, Figure 2E).

Adjuvant chemotherapy (AC) is the primary therapeutical modality for surgically treated CRC patients, especially for those within stage III/IV. Hence, we next made efforts to identify whether *Fn* level is associated with AC benefits in stage III/IV patients. In this study, majority of patients (n=239) received standard AC treatment postoperatively, while the rest (n=41) failed due to some factors such as poor physical condition and financial problems. As shown in Figure 3A, the survival analysis demonstrated that patients receiving AC had a dramatically better CSS and DFS than those receiving no AC (CSS: $p<0.001$; DFS, $p<0.001$). In the subgroups classified by *Fn* level, we found that AC treatment was associated with a significantly better clinical outcome in both patients with low *Fn* level (CSS: $p<0.001$, DFS: $p<0.001$, Figure 3B) and high *Fn* level (CSS: $p=0.034$, DFS: $p=0.024$, Figure 3C). However, the interaction analysis based on factorial design indicated that patients with low *Fn* level benefit more from AC than those with high *Fn* level, in terms of DFS (CSS: $p=0.134$; DFS: $p=0.048$).

Expression and clinical significance of EMT and CSC markers in stage III/IV CRC patients

The representative images of IHC assay are shown in Figure 4. ROC curves were employed to estimate the cutoff values of staining scores for these markers and the results are shown in Figure S1. The cutoff value is 2.5 for E-cadherin and Sox-2, 3.5 for Oct-4, and 5 for N-cadherin and Nanog. Therefore, we used these cutoff values for the following statistical analysis and the correlations between their expression and clinicopathological features are summarized in Table S1. We noted that expression of these markers was significantly correlated with prognosis-related clinical features. For instance, both E-cadherin and N-cadherin expression was correlated with LNM status and distant metastasis (all $p<0.05$).

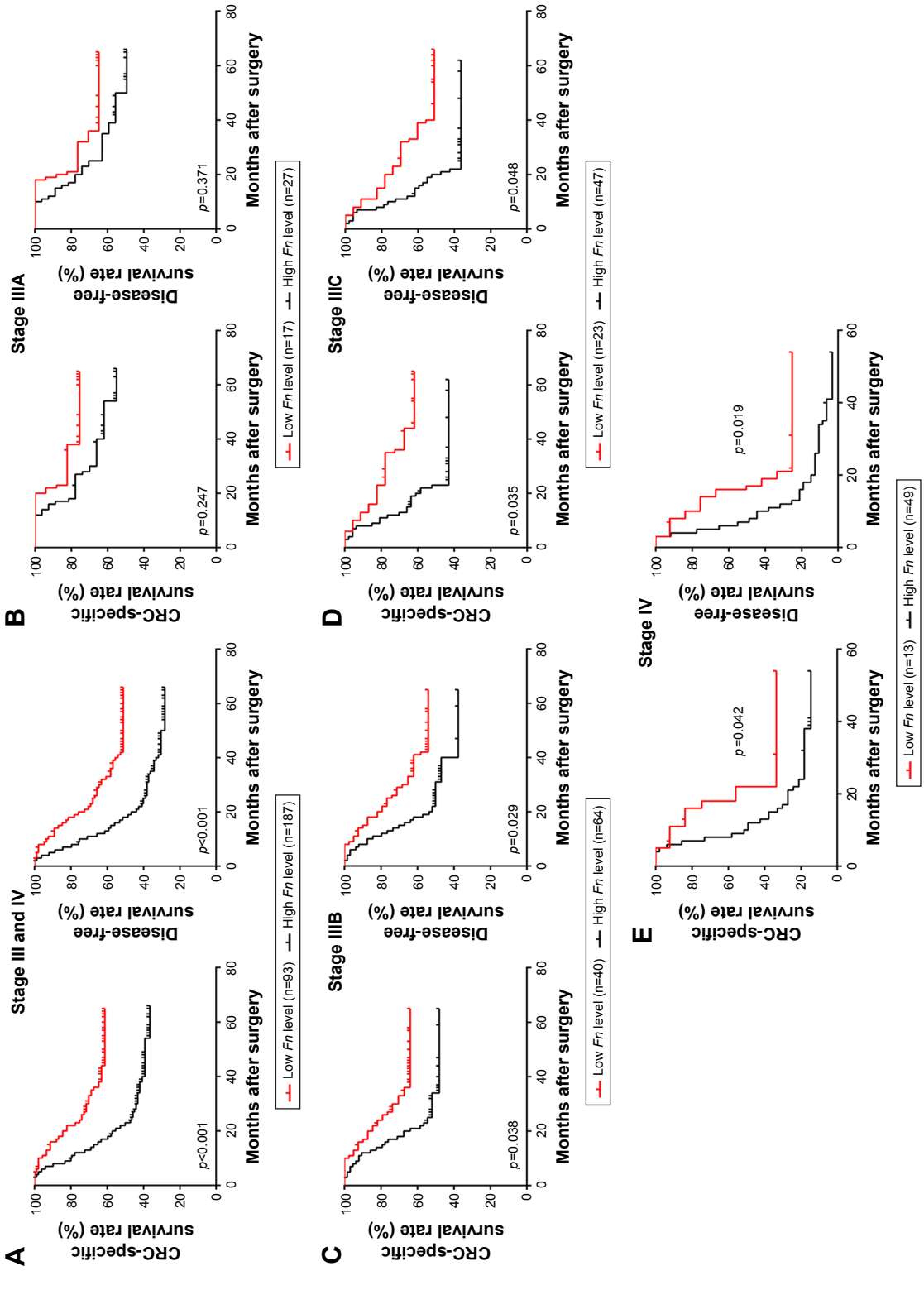

Figure 2 Prognostic significance of *Fn* in stage III/IV CRC patients.
Note: CSS and DFS curves of stage III/IV CRC patients (**A**), stage IIIA patients (**B**), stage IIIB patients (**C**), stage IIIC patients (**D**), stage IV patients (**E**).
Abbreviations: *Fn*, *Fusobacterium nucleatum*; CRC, colorectal cancer; CSS, cancer-specific survival; DFS, disease-free survival.

Table 2 Univariate and multivariate analysis for prognostic factors in cancer-specific survival of stage III/IV CRC patients

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Gender	0.835	0.594–1.175	0.300			
Age	1.351	0.943–1.935	0.101			
Tumor location	1.188	0.843–1.676	0.326			
Tumor size	1.199	0.817–1.757	0.354			
Tumor differentiation	0.731	0.497–1.074	0.110			
Ki-67 positivity	0.914	0.629–1.328	0.638			
Body mass index	0.806	0.597–1.087	0.158			
Tumor invasion	1.595	1.097–2.319	0.015	1.341	0.920–1.955	0.127
Lymph node metastasis	1.426	1.136–1.789	0.002	1.430	1.158–1.766	0.001
Distant metastasis	3.507	2.425–5.071	<0.001	3.243	2.232–4.712	<0.001
Serum CEA level	1.466	1.006–2.136	0.046	1.515	1.038–2.212	0.031
Fn level	2.302	1.541–3.437	<0.001	2.222	1.483–3.329	<0.001

Abbreviations: *Fn*, *Fusobacterium nucleatum*; CRC, colorectal cancer; CEA, carcinoembryonic antigen; HR, hazard ratio; CI, confidence interval.

Naong and Sox-2 expression was correlated with LNM status, while Oct-4 expression was correlated with distant metastasis (all $p<0.05$).

The prognostic significance of EMT and CSC markers was analyzed using Kaplan–Meier survival curves. Patients with high E-cadherin expression had a significantly better CSS and DFS than those with low E-cadherin expression (CSS: $p<0.001$, DFS: $p=0.001$, Figure 5A), while the opposite was true for N-cadherin (CSS: $p<0.001$, DFS: $p<0.001$, Figure 5B), Nanog (CSS: $p<0.001$, DFS: $p<0.001$, Figure 5C), Oct-4 (CSS: $p=0.006$, DFS: $p<0.001$, Figure 5D), and Sox-2 (CSS: $p<0.001$, DFS: $p=0.001$, Figure 5E).

Correlations of *Fn* with EMT and CSC markers in stage III/IV CRC patients

The correlations between *Fn* level and expression of EMT/CSC markers in CRC tissues are summarized in Table 4. *Fn* level was negatively correlated with E-cadherin expression ($r=-0.301$, $p<0.001$), but positively correlated with expression of N-cadherin ($r=0.377$, $p<0.001$) and Nanog ($r=0.362$, $p<0.001$). No significant association was observed between *Fn* level and Sox-2 expression ($r=0.105$, $p=0.078$) or Oct-4 expression ($r=0.099$, $p=0.097$).

Discussion

Fn is a gram-negative anaerobe that is enriched in the oral cavity but hardly detected in other body organs under physiological conditions.[24] However, under pathological conditions, it disseminates and colonizes into extraoral sites to function as pathogenic bacteria for various diseases such as inflammatory bowel disease, organ abscess, and adverse pregnancy outcome.[25–27] In human malignancies, it is perhaps most relevant to CRC, although some emerging evidences have suggested its implication in esophageal and

Table 3 Univariate and multivariate analysis for prognostic factors in disease-free survival of stage III/IV CRC patients

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
Gender	0.821	0.603–1.119	0.212			
Age	1.092	0.796–1.498	0.585			
Tumor location	1.149	0.842–1.568	0.381			
Tumor size	1.132	0.796–1.609	0.490			
Ki-67 positivity	0.998	0.708–1.408	0.993			
Body mass index	0.871	0.665–1.143	0.319			
Tumor differentiation	0.636	0.453–0.893	0.009	0.592	0.417–0.841	0.003
Tumor invasion	1.634	1.163–2.297	0.005	1.499	1.060–2.119	0.022
Lymph node metastasis	1.287	1.050–1.579	0.015	1.294	1.069–1.566	0.008
Distant metastasis	3.965	2.843–5.531	<0.001	3.914	2.788–5.495	<0.001
Serum CEA level	1.512	1.075–2.128	0.018	1.483	1.046–2.101	0.027
Fn level	2.133	1.496–3.041	<0.001	2.000	1.396–2.865	<0.001

Abbreviations: *Fn*, *Fusobacterium nucleatum*; CRC, colorectal cancer; CEA, carcinoembryonic antigen; HR, hazard ratio; CI, confidence interval.

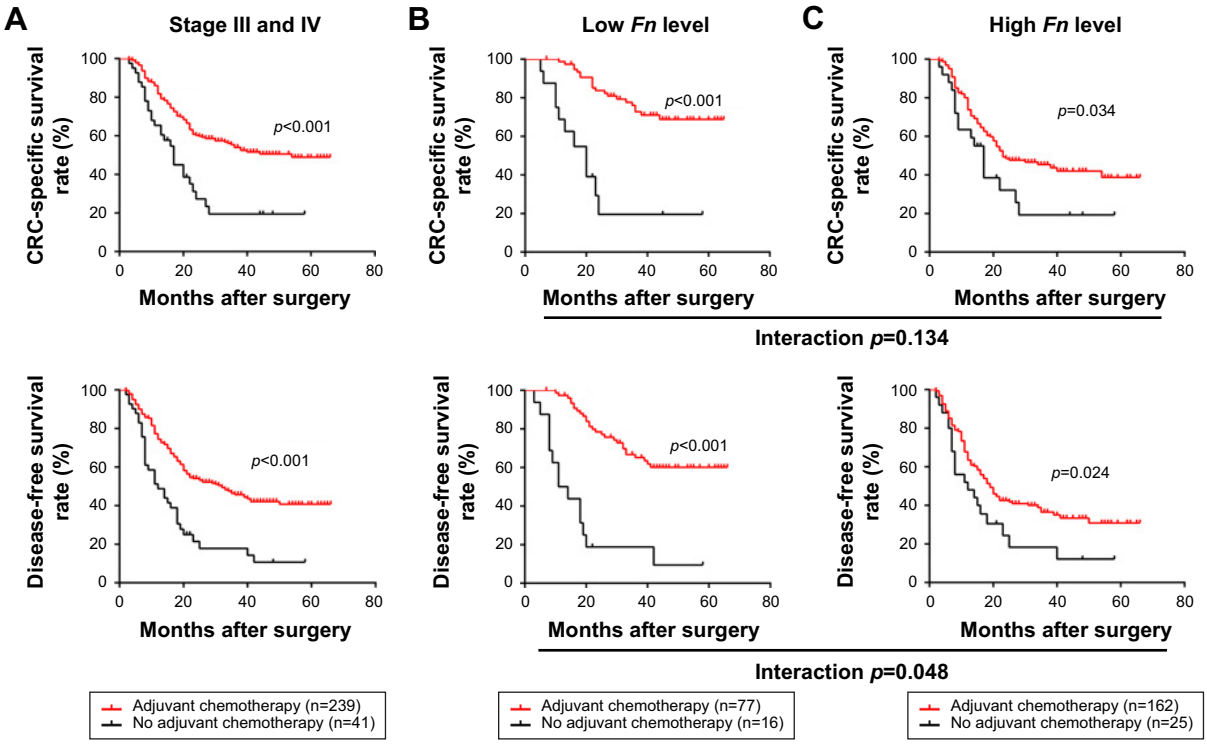

Figure 3 Correlations between *Fn* level and chemotherapy benefits in stage III/IV patients.
Notes: (**A**) CSS and DFS of the whole cohort, (**B**) CSS and DFS of low *Fn* level group, and (**C**) CSS and DFS of high *Fn* level group stratified by chemotherapy reception. An interaction analysis indicates that patients with low *Fn* level benefit more from chemotherapy than those with high *Fn* level, in terms of DFS (CSS: p=0.134; DFS: p=0.048).
Abbreviations: *Fn*, *Fusobacterium nucleatum*; CRC, colorectal cancer; CSS, cancer-specific survival; DFS, disease-free survival.

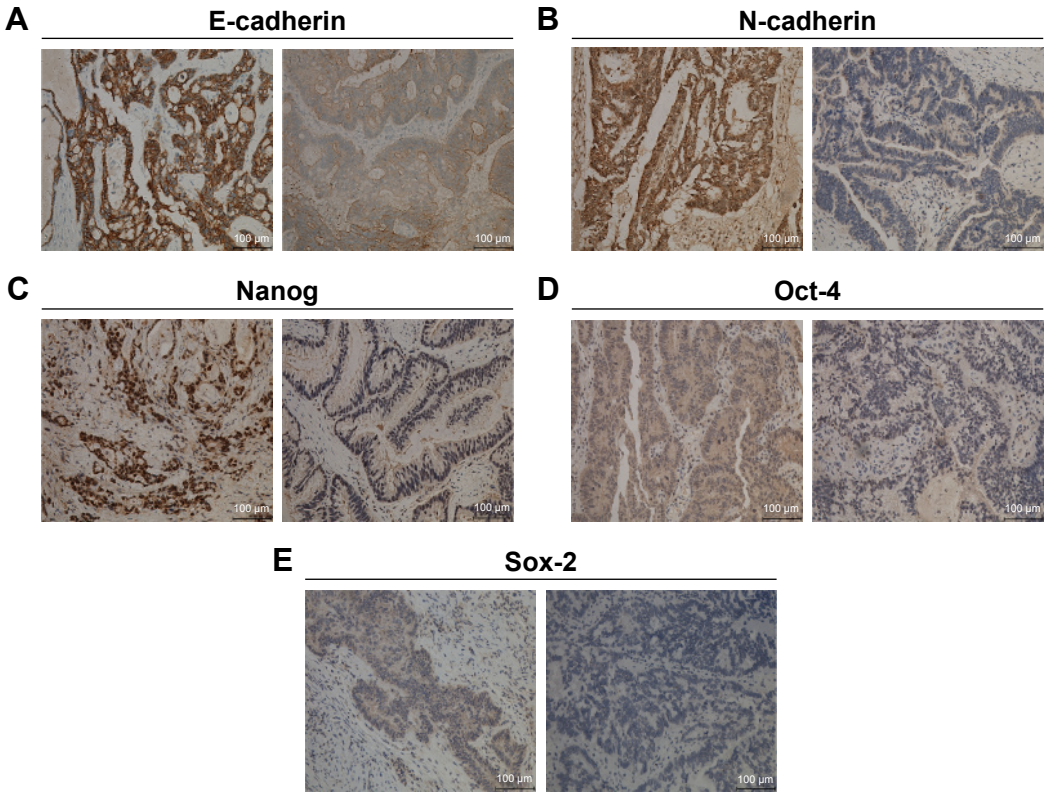

Figure 4 Representative immunohistochemical staining images of EMT and CSC markers in CRC tissues.
Notes: High (left) and low (right) expression of E-cadherin (**A**), N-cadherin (**B**), Nanog (**C**), Oct-4 (**D**), Sox-2 (**E**). Magnification: ×200.
Abbreviations: CRC, colorectal cancer; EMT, epithelial-to-mesenchymal transition; CSC, cancer stem cell.

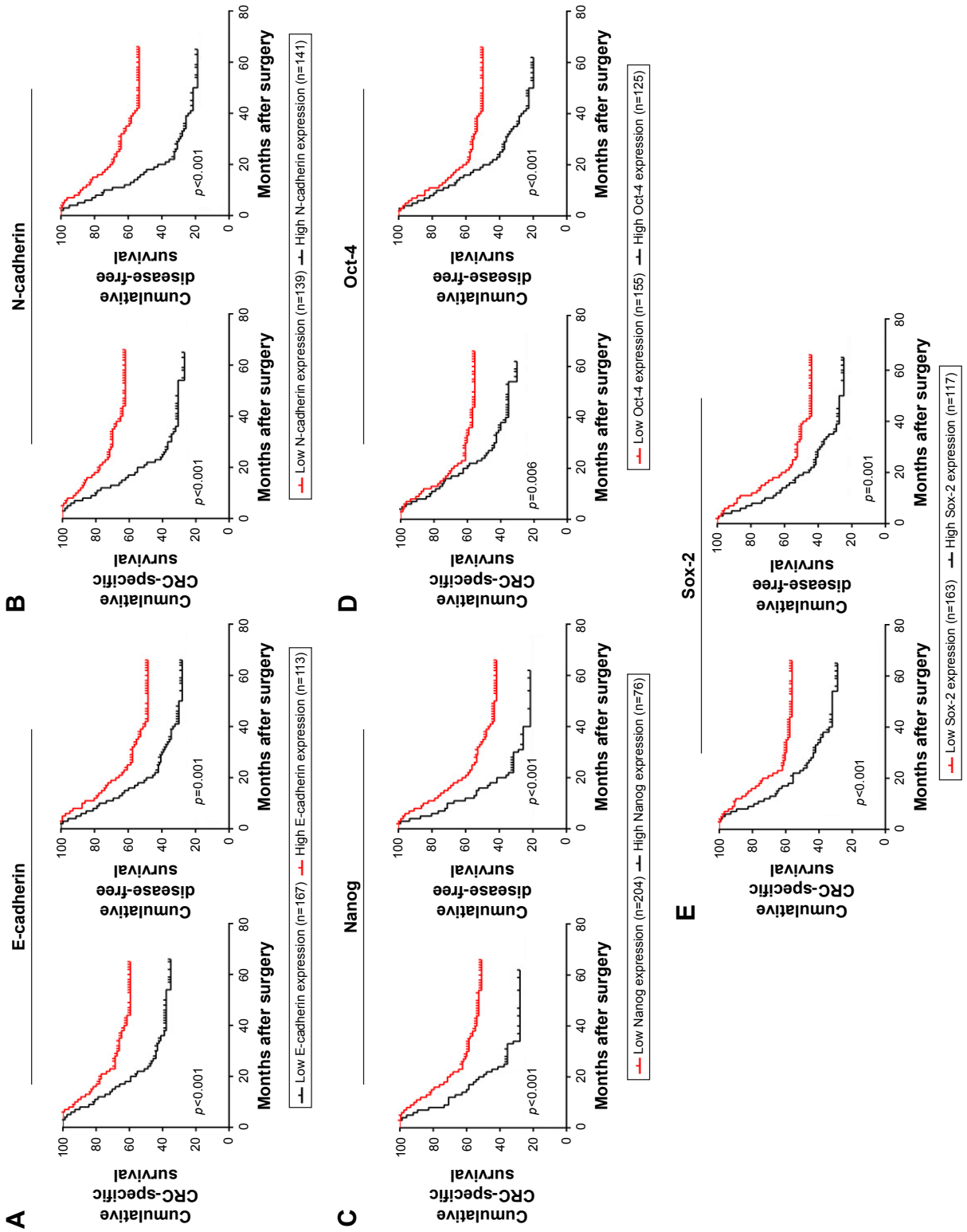

Figure 5 Prognostic significance of epithelial-to-mesenchymal transition and cancer stem cell markers in stage III/IV CRC patients.
Note: CSS and DFS curves stratified by E-cadherin expression (**A**), N-cadherin expression (**B**), Nanog expression (**C**), Oct-4 expression (**D**), Sox-2 expression (**E**).
Abbreviations: CRC, colorectal cancer; CSS, cancer-specific survival; DFS, disease-free survival.

Table 4 Correlations of *Fn* with EMT/CSC markers in stage III/IV CRC patients

Markers	N	*Fn* level		*r*	*p*-value
		Low	High		
E-cadherin					<0.001
Low	167	36	131	−0.301	
High	113	57	56		
N-cadherin					<0.001
Low	139	71	68	0.377	
High	141	22	119		
Sox-2					0.078
Low	163	61	102	0.105	
High	117	32	85		
Oct-4					0.097
Low	155	58	97	0.099	
High	125	35	90		
Nanog					<0.001
Low	204	89	115	0.362	
High	76	4	72		

Abbreviations: *Fn*, *Fusobacterium nucleatum*; CRC, colorectal cancer; EMT, epithelial-to-mesenchymal transition; CSC, cancer stem cell.

pancreatic cancer.[28,29] Using RNA sequencing, Castellarin et al for the first time proposed that *Fn* infection might be prevalent in CRC patients.[30] Then, increasing studies made efforts to investigate its potential oncogenic mechanisms in CRC where its regulatory role in tumor immunity is the most extensively discussed.[31–33] In addition, *Fn* is found to be abundant in premalignant lesions with positive CpG island methylator phenotype, implying its involvement in epigenetic changes of early tumorigenesis.[34] However, despite these novel findings about its oncogenic role, its prognostic significance in CRC patients remains unclear and whether it has the potential utility for improving the current TNM-based prognostic system still needs to be validated.

In this study, the level and clinical significance of *Fn* were analyzed in a cohort of 280 surgically treated stage III/IV patients. Firstly, we found that the *Fn* level is significantly higher in tumor tissues than that in adjacent normal tissues in both stage III and IV patients, supporting its promoting role in CRC initiation and development. A recent study proposed that this promoting role may be partly attributed to its participation in oncogenic biofilm formation.[35] The following correlation analysis demonstrated that *Fn* level is significantly correlated with tumor invasion, LNM status, and distant metastasis. This further confirmed our previous finding that *Fn* enhances the malignant characteristics of CRC cells in vitro and in vivo.[19] Li et al proved that *Fn* level is positively associated with the presence of LNM but not with tumor invasion in a relatively smaller cohort of CRC patients (n=101), partly consistent with our present result.[36] Furthermore, Castellarin et al found that 74.4% (29/39) of CRC patients with high *Fn* level had positive LNM as compared with 44.8% (26/58) of those with low *Fn* level, also indicating a close correlation between *Fn* and LNM.[30] Therefore, given these evidences, we concluded that *Fn* level might be a promising indicator for CRC metastasis in CRC patients, especially for those with positive LNM.

Although *Fn* level has been identified as an unfavorable prognostic factor in several studies, its specific prognostic significance for stage III/IV patients remains unknown.[37,38] Using the Kaplan–Meier model, our survival analysis showed that stage III/IV patients with high *Fn* level had a significantly worse CSS and DFS than those with low *Fn* level. The following univariate and multivariate analysis not only further confirmed a significant correlation between *Fn* level and patient survival, but also revealed its independence in prognosis prediction. Given these results, we preliminarily proposed that *Fn* level might serve as a predictor for clinical outcome of stage III/IV patients. Several studies have recently suggested the limitation of traditional LNM status in prognosis stratification of stage III patients, strongly urging us to investigate whether the *Fn* level has the capacity to provide an accurate stratification for these patients.[39,40] We therefore subsequently performed a subgroup analysis and found that *Fn* level could stratify the CSS and DFS of both stage IIIB and IIIC patients, but failed in stage IIIA patients. This result suggested that *Fn* level might be an effective prognostic indicator only for stage IIIB or IIIC patients. We also speculate that this result is partly attributed to the survival paradox that stage IIIA patients, clinically characterized as $T_{1-2}N_{1-2a}M_0$, have a significantly better prognosis than other stage III and even most stage II patients, with a 5-year overall survival rate ranging from 81.6% to 85.6% as reported.[41,42] This abnormally favorable prognosis may contribute to the failed prognostic stratification of *Fn* level in stage IIIA patients and we therefore suggest that detecting the *Fn* level in these patients may provide limited beneficial information for clinical management. Furthermore, we found that high *Fn* level is associated with worse outcome in stage IV patients despite the limited samples, implying its potential to be a prognostic predictor for surgically treated patients with distant metastasis. Finally, it should be noted that our study was unable to investigate the prognostic value of fecal *Fn* level in CRC patients, although its diagnostic potential has been highly advocated in several previous studies.[43,44] Hence, whether its fecal level has any prognostic value or serves as a dynamic noninvasive marker like CEA in CRC surveillance still requires our extensive clinical validations in future.

Increasing evidences have supported that gut bacteria play a major role in modulating the anticancer efficacy of

various CRC-related chemotherapeutic drugs such as 5-Fu, irinotecan, and oxaliplatin.[45] To identify the correlation between *Fn* level and chemotherapy benefits in stage III/IV patients, a subgroup analysis was carried out based on *Fn* level and we found that patients receiving chemotherapy had a significantly better prognosis than those receiving no chemotherapy in both the high and low *Fn* level group. However, the following interaction analysis on DFS indicated that patients with low *Fn* level benefited more from chemotherapy than those with high *Fn* level, suggesting that *Fn* might be a predictive biomarker for chemotherapy response in stage III/IV patients. These results also implied its potential involvement in chemotherapy resistance of metastatic CRC cells. Yu et al have recently found that *Fn* can induce chemotherapy resistance of CRC cells through modulating autophagy via toll-like receptor/microRNAs signaling cascade, strongly supporting our results.[46] Furthermore, it is reported that chemotherapy may in turn influence the gut bacteria of cancer patients.[47] Therefore, whether the *Fn* level is changed during chemotherapy treatment and this change has any impact on therapy efficacy or even drug toxicity is also worthy of further investigation.

Finally, we analyzed the expression and clinical significance of EMT and CSC markers in stage III/IV patients, based on the consideration that both the molecular events play a major part in disease progression and therapy resistance of cancer patients.[48] Our results showed that these markers are correlated with not only some clinicopathological features, but also CSS and DFS in stage III/IV patients. These findings are consistent with those of previous studies regarding their clinical significance in CRC patients.[49–51] More importantly, through correlation analysis, we found that the *Fn* level was negatively correlated with E-cadherin expression, but positively correlated with N-cadherin expression in CRC tissues. Since loss of E-cadherin and gain of N-cadherin are defined as classical hallmarks of EMT, we speculated that *Fn* might contribute to CRC development partly by inducing this oncogenic molecular phenotype.[52] This speculation is partly supported by a recent study that proved that *Fn* promotes CRC growth and invasion through regulating E-cadherin/β-catenin signaling.[53] Our previous study also found that *Fn* upregulates miR-21 level to induce colitis-associated cancer by repressing E-cadherin, implying that *Fn* may induce EMT through upregulating miR-21.[19,54] Then, we observed a positive correlation between *Fn* level and Nanog expression in CRC tissues, indicating that *Fn* might be involved in CSC phenotype. Nanog, as a well-established CSC marker, is also found to participate in the EMT program in cancer development, suggesting that *Fn* may partly induce EMT through regulating CSC phenotype.[55,56] However, for further clarifying the correlation of *Fn* with EMT and CSC phenotype, extensive cellular assays are needed. In addition, it is reported that statins enhance the chemosensitivity of CRC cells through impairing CSC phenotype and whether *Fn* screening may be useful to discriminate between patients who most likely benefit from statins during chemotherapy still requires more clinical validations.[57]

In summary, our study indicates that *Fn* level is positively correlated with malignant progression and may serve as an independent prognostic indicator in stage III/IV patients. In addition, our findings also suggest that the *Fn* level is helpful for predicting chemotherapy benefits in these patients. Finally, we found that *Fn* level is correlated with several EMT and CSC markers in their tumor tissues, suggesting its potential involvement in EMT-CSC cross talk during CRC development. These findings not only suggest the immense potential of *Fn* as a clinically actionable biomarker for precise treatment in stage III/IV patients, but also provide a promising adjuvant therapeutic strategy for them that targeting *Fn* may be helpful for preventing CRC metastasis and improving chemotherapy efficacy.

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

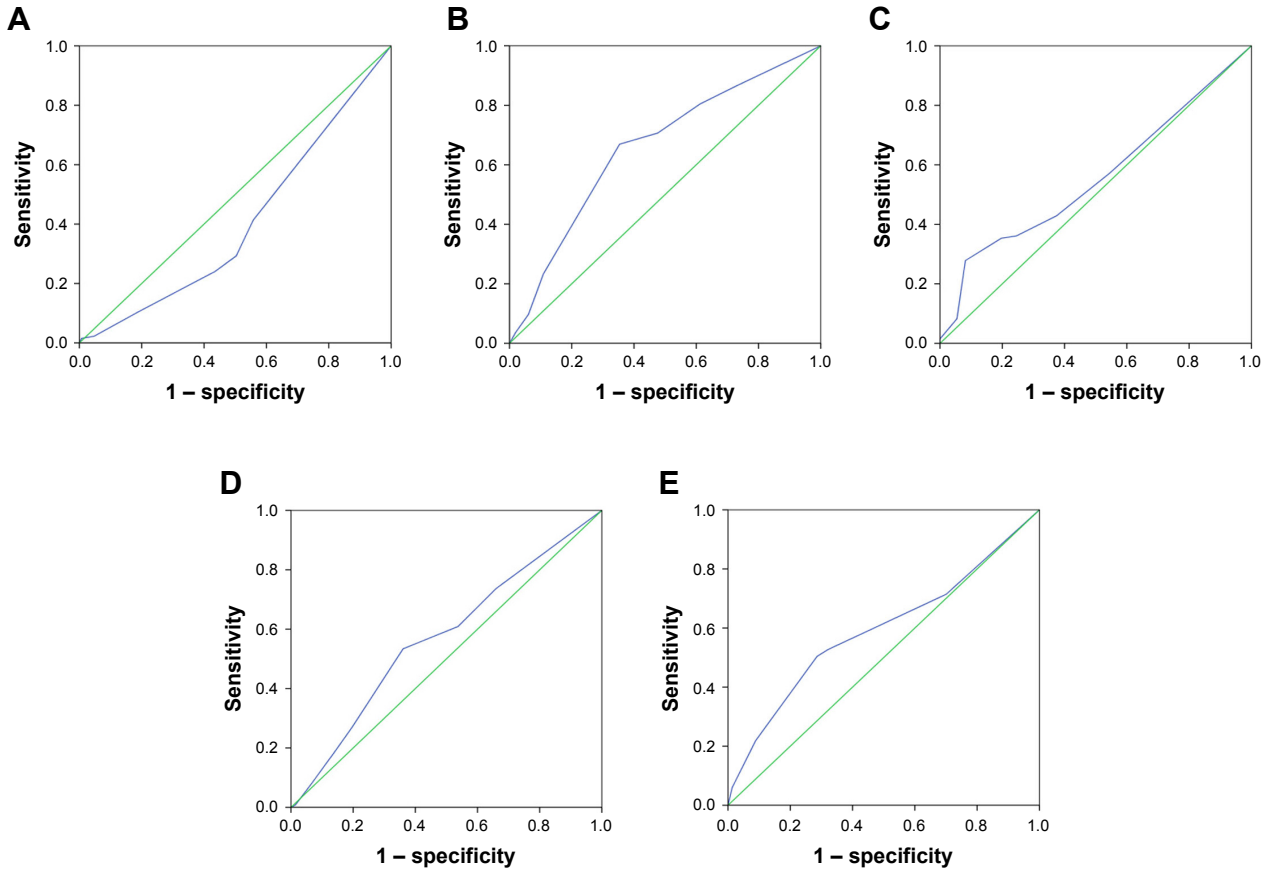

Figure S1 The ROC curve analysis is used to determine the cutoff values of staining scores of epithelial–mesenchymal transition and cancer stem cell markers.
Notes: (**A**) E-cadherin; (**B**) N-cadherin; (**C**) Nanog; (**D**) Oct-4; (**E**) Sox-2.
Abbreviation: ROC, receiver operating characteristic.

Table S1 Correlations between epithelial-to-mesenchymal transition/cancer stem cell markers and clinicopathological characteristics

Characteristics	Total	E-cadherin		*p*-value	Total	N-cadherin		*p*-value	Total	Sox-2		*p*-value	Total	Oct-4		*p*-value	Total	Nanog		*p*-value
		Low	High			Low	High			Low	High			Low	High			Low	High	
Gender				0.761				0.892				0.621				0.279				0.434
Female	122	74	48		122	60	62		122	69	53		122	72	50		122	86	36	
Male	158	93	65		158	79	79		158	94	64		158	83	75		158	118	40	
Age				0.960				0.607				0.878				0.892				0.559
≤65 years	111	66	45		111	53	58		111	64	47		111	62	49		111	83	28	
>65 years	169	101	68		169	86	83		169	99	70		169	93	76		169	121	48	
Tumor location				0.708				0.117				0.748				0.803				0.538
Rectal	130	76	54		130	58	72		130	77	53		130	73	57		130	97	33	
Colon	150	91	59		150	81	69		150	86	64		150	82	68		150	107	43	
Tumor size				0.297				0.233				0.904				0.664				0.731
≤5 cm	214	124	90		214	102	112		214	125	89		214	120	94		214	157	57	
>5 cm	66	43	23		66	37	29		66	38	28		66	35	31		66	47	19	
Tumor differentiation				0.555				0.311				0.422				0.992				0.134
Poor	74	42	32		74	33	41		74	46	28		74	41	33		74	49	25	
Well/moderate	206	125	81		206	106	100		206	117	89		206	114	92		206	155	51	
Tumor invasion				0.275				0.555				0.630				0.120				0.010
T1–T2	96	53	43		96	50	46		96	54	42		96	47	49		96	79	17	
T3–T4	184	114	70		184	89	95		184	109	75		184	108	76		184	125	59	
Lymph node metastasis				0.029				0.024				0.046				0.609				0.003
N1	81	39	42		81	31	50		81	43	38		81	45	36		81	59	22	
N2a	95	58	37		95	56	39		95	65	30		95	56	39		95	80	15	
N2b	104	70	34		104	52	52		104	55	49		104	54	50		104	65	39	
Distant metastasis				0.039				0.012				0.396				<0.001				0.118
Absent	218	123	95		218	117	101		218	124	94		218	134	84		218	154	64	
Present	62	44	18		62	22	40		62	39	23		62	21	41		62	50	12	
Ki-67 positivity				0.042				0.382				0.483				0.394				0.041
<30%	78	54	24		78	42	36		78	48	30		78	40	38		78	50	28	
≥30%	202	113	89		202	97	105		202	115	87		202	115	87		202	154	48	
Serum CEA level				0.199				0.971				0.174				0.764				0.276
≤5 ng/mL	99	54	45		99	49	50		99	63	36		99	56	43		99	76	23	
>5 ng/mL	181	113	68		181	90	91		181	100	81		181	99	82		181	128	53	
BMI				0.472				0.887				0.367				0.394				0.166
<18.5 kg/m^2	22	12	10		22	12	10		22	14	8		22	11	11		22	13	9	
18.5–24.99 kg/m^2	178	111	67		178	88	90		178	98	80		178	104	74		178	128	50	
≥25.0 kg/m^2	80	44	36		80	39	41		80	51	29		80	40	40		80	63	17	

Abbreviations: CEA, carcinoembryonic antigen; BMI, body mass index.