

# Human Microbiome *Fusobacterium Nucleatum* in Esophageal Cancer Tissue Is Associated with Prognosis

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## Abstract

**Purpose:** *Fusobacterium nucleatum* (*F. nucleatum*) is a component of the human microbiome that primarily inhabits the oral cavity. It causes periodontal disease and has also been implicated in the development of human cancers. Although there are several reports of the relationship between *F. nucleatum* and the clinical outcome in human cancers, its prognostic significance in esophageal cancer remains unclear.

**Experimental Design:** We quantified *F. nucleatum* DNA in 325 resected esophageal cancer specimens by qPCR. Significant pathways in *F. nucleatum*-positive esophageal cancer tissues were identified by Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis using microarray data.

**Results:** Esophageal cancer tissues contained significantly more *F. nucleatum* DNA than matched normal esophageal mucosa ( $P = 0.021$ ;  $n = 60$ ). *F. nucleatum* DNA was detected in 74 of 325 cases

(23%). *F. nucleatum* DNA positivity was significantly associated with tumor stage, but not with sex, age, performance status, tobacco use, alcohol use, histology, tumor location, or preoperative treatment. *F. nucleatum* DNA positivity was also significantly associated with cancer-specific survival [log-rank  $P = 0.0039$ ; univariate HR = 2.01; 95% confidence interval (CI), 1.22–3.23;  $P = 0.0068$ ; multivariate HR = 1.78; 95% CI, 1.06–2.94;  $P = 0.031$ ]. The top-ranked KEGG pathway in *F. nucleatum*-positive tissues was "cytokine–cytokine receptor interaction." A significant relationship between *F. nucleatum* and the chemokine CCL20 was validated by IHC.

**Conclusions:** *F. nucleatum* in esophageal cancer tissues was associated with shorter survival, suggesting a potential role as a prognostic biomarker. *F. nucleatum* might also contribute to aggressive tumor behavior through activation of chemokines, such as CCL20. *Clin Cancer Res*; 22(22); 5574–81. ©2016 AACR.

## Introduction

Esophageal cancer is the fifth most common cause of cancer-related death in men and the eighth most common in women worldwide (1). Despite the development of multimodal therapies, including surgery, chemotherapy, radiotherapy, and chemoradiotherapy, the prognosis of patients, including those who undergo complete resection, remains poor (2–4). Further studies are therefore needed to clarify the pathogenesis of esophageal cancer and to explore new diagnostic and therapeutic possibilities. In addition, the identification of new prognostic or predictive markers for esophageal cancer could improve the use of risk-

adapted treatment strategies and help to stratify patients for drugs targeting these tumor characteristics in future clinical trials.

Research into the microbiome is a rapidly advancing field in human cancers (5–7). More than 100 trillion bacteria inhabit the human body and form their own flora (microbiome) in individual organs. The gut microbiome has recently been shown to play an important role in health, as well as in diseases, such as obesity (8, 9), inflammatory bowel disease (10, 11), diabetes (12, 13), nonalcoholic fatty liver disease (14, 15), and several types of cancers. *Fusobacterium nucleatum* (*F. nucleatum*; a non-spore-forming, anaerobic gram-negative bacterium) is part of the normal flora in the human oral cavity, vagina, and gastrointestinal mucosa (16). It is recognized as a pathogen in periodontal diseases (17), chorioamnionitis (18), and inflammatory bowel disease (10, 19). Regarding the association between *F. nucleatum* and gastrointestinal cancers, metagenomic analysis has shown an overabundance of *F. nucleatum* in colorectal cancer tissues (20, 21). *F. nucleatum* has also been shown to activate the WNT/ $\beta$ -catenin signaling pathway in colorectal cancer cells, and to potentially promote tumor growth (22). Recent studies reported that high levels of *F. nucleatum* DNA were linked to a poor prognosis in human cancers (23, 24), whereas others reported no association between *F. nucleatum* DNA levels and patient survival (Supplementary Table S1; refs. 20, 25). However, no studies to date have examined the prognostic impact of *F. nucleatum* in esophageal cancer tissues.

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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### Translational Relevance

*Fusobacterium nucleatum* (*F. nucleatum*), which primarily inhabits the oral cavity, causes periodontal disease. *F. nucleatum* influences the development and progression of colorectal cancer. Furthermore, the presence of *F. nucleatum* is associated with a poor prognosis in patients with colorectal cancer. However, no studies to date have examined the prognostic impact of *F. nucleatum* in esophageal cancer. In this study, we quantified *F. nucleatum* DNA in 325 resected esophageal cancer specimens by qPCR. This is the first study to provide the evidence for the relationship between *F. nucleatum* and poor prognosis in esophageal cancer. In addition, using KEGG enrichment analysis, we demonstrated that *F. nucleatum* might contribute to the acquisition of aggressive tumor behavior through the activation of chemokines, such as CCL20. Our data suggest that *F. nucleatum* DNA status can have a potential role as a prognostic biomarker.

In this study, we quantified *F. nucleatum* DNA in 325 samples of resected esophageal cancer by qPCR and examined its prognostic value. We also clarified the mechanism whereby *F. nucleatum* may confer a poor prognosis by enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. The results of this study suggest that *F. nucleatum* may have a potential role as a prognostic biomarker in esophageal cancer.

## Materials and Methods

### Study group

We analyzed 325 formalin-fixed, paraffin-embedded (FFPE) esophageal cancer specimens from consecutive patients undergoing resection at Kumamoto University Hospital (Kumamoto, Japan) between April 2005 and June 2013. Tumor staging was carried out according to the American Joint Committee on Cancer Staging Manual (7th edition; ref. 26). The vast majority of cases were diagnosed as squamous cell carcinoma (SCC); there were 300 cases (92%) of SCC, 12 (3.7%) of adenocarcinoma, and 13 (4.0%) of others. A total of 117 received preoperative treatment [73 chemotherapy (cisplatin, 5-fluorouracil either with or without docetaxel) 44 chemoradiotherapy]. Patients were observed at 1- to 3-month intervals until death or January 31, 2016, whichever came first. Overall survival (OS) was defined as the time from the date of surgery to the date of death. Cancer-specific survival was defined as the time from the date of surgery and the date of death attributable to esophageal cancer. Written informed consent was obtained from each subject, and the study procedures were approved by the Institutional Review Board. The term "prognostic marker" is used throughout this article according to the REMARK guidelines (27).

### DNA extraction and qPCR for *F. nucleatum*

Genomic DNA was extracted from FFPE esophageal cancer tissues using a QIAamp DNA FFPE Tissue Kit (Qiagen). We determined the amount of *F. nucleatum* DNA by qPCR assay. The *nus G* gene of *F. nucleatum* and the reference human gene *SLCO2A1* were amplified using custom-made TaqMan primer/probe sets (Applied Biosystems). The primer and probe sequences for each Custom TaqMan Gene Expression Assay were

as follows: *F. nucleatum* forward primer, 5'-TGGTGCATTCTTC-CAAAAATATCA-3'; *F. nucleatum* reverse primer, 5'-AGATCAA-GAAGGACAAGTTGCTGAA-3'; *F. nucleatum* FAM probe, 5'-AC-TTAACTCTACCATGTTCA-3'; *SLCO2A1* forward primer, 5'-ATCCCCAAAGCACCTGGTTT-3'; *SLCO2A1* reverse primer, 5'-AGAGGCCAAGATAGTCCTGGTAA-3'; *SLCO2A1* VIC probe, 5'-CCATCCATGTCCTCATCTC-3'. Assays were performed in a 384-well optical PCR plate. DNA was amplified and detected using a LightCycler 480 Instrument II (Roche) under the following reaction conditions: initial denaturation at 95°C for 10 minutes, 15 seconds at 95°C, and 60 seconds at 60°C. The amount of *F. nucleatum* DNA in each tissue was normalized relative to *SLCO2A1* (28).

### Immunohistochemical staining

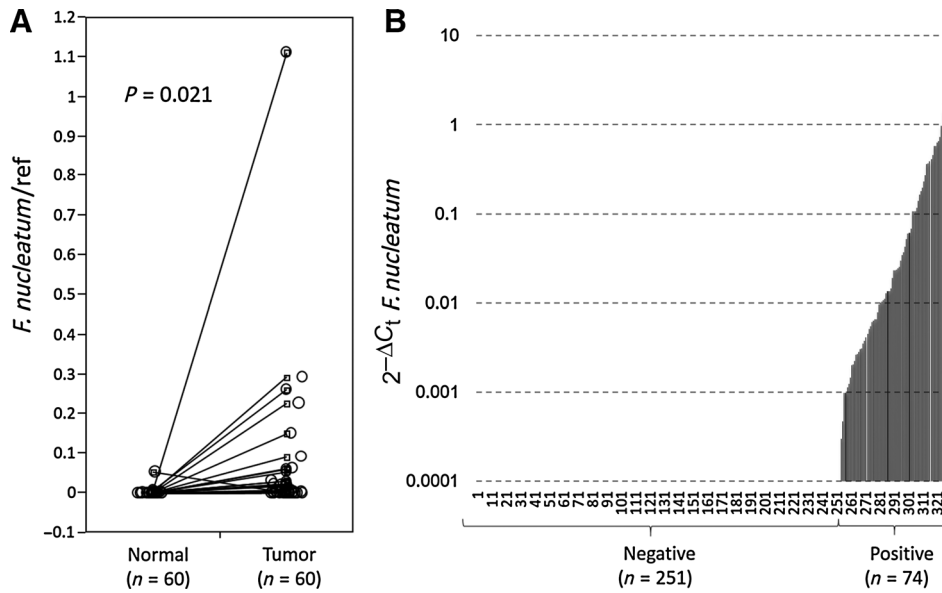
FFPE tissue was serially sectioned at 3 to 5  $\mu$ m, dewaxed, deparaffinized in xylene, and rehydrated through a series of graded alcohols. The samples were boiled for 15 minutes in a microwave oven in Histofine (pH = 9.0; Nichirei) to increase antigen retrieval. Endogenous peroxidases were blocked by 3% hydrogen peroxidase treatment for 30 minutes. The slides were incubated with primary antibody (1:50 dilution of rabbit mAb for macrophage inflammatory protein 3 $\alpha$  (CC-chemokine cysteine motif chemokine ligand 20, CCL20; ab9829; Abcam) overnight at 4°C. Detection was performed with a biotin-free horseradish peroxidase-labeled polymer of the Envision Plus detection system (Dako). The sections were developed in 3,3-diaminobenzidine and counterstained with Mayer hematoxylin. The slides were then dehydrated through graded alcohols and covered with coverslips. Staining intensity and percentage of CCL20-positive tumor cells were assessed. The extent of staining was categorized semiquantitatively, based on the percentage of positive tumor cells: 0 ( $\leq$ 5% positive cells), 1 (6%–25% positive cells), 2 (26%–50% positive cells), 3 (51%–75% positive cells), and 4 (>75% positive cells). The intensities of cytoplasmic and membrane staining were also determined semiquantitatively as follows: 0 (negative), 1 (weakly positive), 2 (moderately positive), and 3 (strongly positive). The scores of sections were defined as "extent of staining  $\times$  intensity."

### Microarray and KEGG pathway enrichment analysis

Total RNA was isolated from frozen sections of 10 esophageal cancer biopsy specimens using an RNeasy Mini Kit (Qiagen). Gene expression microarray analysis was carried out using SurePrint G3 Human GE Microarray 8  $\times$  60K Ver. 2.0 (Agilent Technologies) according to the manufacturer's protocol. Differentially expressed genes (DEG) were screened by comparing the RNA expression levels of esophageal cancer specimens between *F. nucleatum*-positive and *F. nucleatum*-negative groups, using Subio Platform (Subio Inc.). KEGG pathway enrichment analyses were performed to identify the biological functions and pathways represented by the identified DEGs using the Database for Annotation, Visualization and Integrated Discovery 6.7 software (<https://david.ncifcrf.gov/>; ref. 29).

### Statistical analysis

All statistical analyses were carried out using JMP, version 10 (SAS Institute). All *P* values were two-sided. We compared mean values using Student *t* tests for age and body mass index, and  $\chi^2$  or Fisher exact tests for all other variables. Survival time distribution in the survival analysis was assessed by the



**Figure 1.** *F. nucleatum* expression in esophageal cancer. **A**, *F. nucleatum* expression in tumor and adjacent normal tissue samples in 60 patients with esophageal cancer. *F. nucleatum* expression was significantly higher in tumor than in adjacent normal tissue ( $P = 0.021$ ). **B**, *F. nucleatum* expression status in 325 patients with esophageal cancer.

Kaplan–Meier method using log-rank tests. We constructed a multivariate model to compute the HR based on the *F. nucleatum* DNA status, including sex (male vs. female), age at surgery (<65 vs. ≥65 years), year of surgery (2005–2009 vs. 2010–2013), tobacco use (yes vs. no), alcohol use (yes vs. no), performance status (0 vs. 1–), tumor location (upper vs. lower), tumor stage (I and II vs. III and IV), and preoperative treatment (absent vs. present). Interactions were assessed by including the cross-product of the *F. nucleatum* DNA status and another variable of interest in a Cox model.

**Results**

***F. nucleatum* in esophageal cancer tissues**

We assessed the relative amounts of *F. nucleatum* DNA in esophageal cancer tissues by qPCR assay. *F. nucleatum* DNA levels were higher in esophageal cancer tissues than in paired adjacent nontumor tissues ( $n = 60$ ,  $P = 0.021$ ; Fig. 1A). We also measured the relative *F. nucleatum* DNA levels in cancer tissues from the 325 esophageal cancer cases. *F. nucleatum* was detected in 74 (23%) of 325 cases (Fig. 1B). The relative *F. nucleatum* DNA content in esophageal cancer tissues ranged from  $3.0 \times 10^{-4}$ – $2.8 \times 10^0$  (median,  $2.3 \times 10^{-2}$ ).

**Relationship between tumor *F. nucleatum* DNA status and clinicopathologic features in esophageal cancer**

The clinicopathologic features of the 325 cases according to *F. nucleatum* status are shown in Table 1. *F. nucleatum* positivity was not associated with age, sex, year of operation, preoperative performance status, smoking history, alcohol history, comorbidity, tumor location, histology, tumor size, or preoperative therapy (all  $P > 0.05$ ), but was associated with tumor stage ( $P = 0.016$ ), T stage ( $P < 0.01$ ), and N stage ( $P = 0.039$ ).

**Tumor *F. nucleatum* DNA status and patient survival**

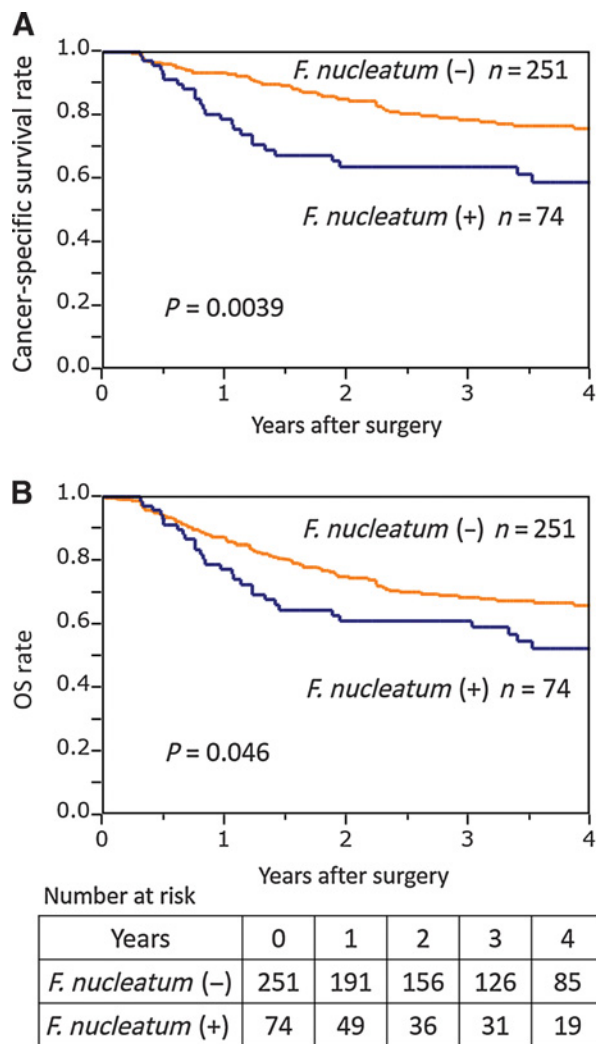
There were 112 deaths among the 325 esophageal cancer patients, including 75 esophageal cancer-specific deaths. The median follow-up time for censored patients was 2.6 years.

**Table 1.** *F. nucleatum* DNA status in esophageal cancers and clinical and tumor features

Clinical or pathologic feature	N	<i>F. nucleatum</i> DNA		P
		Negative	Positive	
All cases	325	251	74	
Mean age ± SD	65.9 ± 9.2	65.6 ± 9.5	66.7 ± 8.0	0.52
Sex				1.0
Male	287	221	66	
Female	38	30	8	
Year of operation				0.69
2005 to 2009	172	131	41	
2010 -	153	120	33	
Performance status				1.0
0	252	194	58	
1 -	73	57	16	
Tobacco use				0.86
Yes	271	210	61	
No	54	41	13	
Alcohol use				0.72
Yes	274	210	64	
No	51	41	10	
Comorbidity				0.48
Present	223	175	48	
Absent	102	76	26	
Location				0.16
Upper	54	46	8	
Lower	271	205	66	
Histology				0.64
SCC	300	230	70	
Adeno	12	10	2	
Others	13	11	2	
Tumor size ± SD	4.2 ± 3.5	3.9 ± 2.4	4.4 ± 1.8	0.12
Stage				0.016
I	155	131	24	
II	42	32	10	
III	118	82	36	
IV	10	6	4	
Preoperative treatment				0.053
Present	117	83	34	
Absent	208	168	40	

Abbreviation: Adeno, adenocarcinoma.

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**Figure 2.** Kaplan-Meier curves for cancer-specific survival (A) and OS (B) in patients with esophageal cancer according to *F. nucleatum* DNA status in tumor tissues.

According to Kaplan-Meier analysis, *F. nucleatum*-positive patients had significantly shorter cancer-specific survival (log-rank  $P = 0.0039$ ) and OS (log-rank  $P = 0.046$ ) compared with *F. nucleatum*-negative cases (Fig. 2). We also analyzed *F. nucleatum* DNA status by Cox regression analysis. *F. nucleatum*-positive

patients had significantly higher cancer-specific mortality compared with *F. nucleatum*-negative patients [HR = 2.01; 95% confidence interval (CI), 1.22–3.23;  $P = 0.0068$ ; Table 2]. In the multivariate Cox model adjusted for clinical, pathologic, and epidemiologic features, *F. nucleatum* positivity was associated with significantly higher cancer-specific mortality (multivariate HR = 1.78; 95% CI, 1.06–2.94;  $P = 0.032$ ; Table 2). Similar results were observed for overall mortality.

**Survival analyses of interaction between *F. nucleatum* and other variables**

We determined whether the influence of *F. nucleatum* on cancer-specific survival was modified by any of the clinical, pathologic, or epidemiologic variables evaluated. The effect of *F. nucleatum* was not significantly modified by age, year of surgery, performance status, tumor location, preoperative treatment, tumor size, or tumor stage (all  $P > 0.09$ ; Fig. 3). Notably, we did not observe a modifying effect of the preoperative treatment on the relationship between *F. nucleatum* and cancer-specific survival rate ( $P_{\text{interaction}} = 0.58$ ).

**Tumor *F. nucleatum* DNA status and patient survival in esophageal SCC**

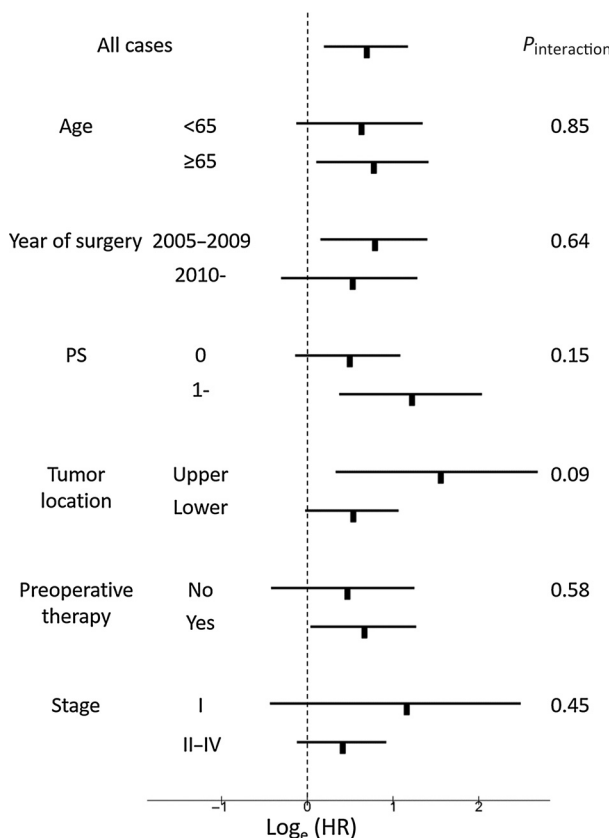
SCC is the predominant type of esophageal cancer in the East, including Japan. We therefore also performed survival analyses, including only SCC ( $n = 300$ ). *F. nucleatum*-positive patients with SCC had significantly lower cancer-specific survival compared with *F. nucleatum*-negative patients (log-rank  $P = 0.0012$ , univariate HR = 2.26; 95% CI, 1.34–3.72;  $P = 0.0026$ ; multivariate HR = 1.98; 95% CI, 1.14–3.37;  $P = 0.016$ ).

**Upregulated pathways in *F. nucleatum*-positive esophageal cancer tissues**

To clarify the mechanism whereby *F. nucleatum* may confer a poor prognosis, we performed enrichment analyses of KEGG pathways using the microarray data. The top 10 most enriched KEGG pathways associated with the significantly upregulated DEGs in *F. nucleatum*-positive esophageal cancer tissues are shown in Fig. 4A. Importantly, "cytokine-cytokine receptor interaction" was the top-ranked pathway (FDR < 0.001, fold enrichment > 1.95). A list of the chemokine DEGs (fold change > 2) in "cytokine-cytokine receptor interaction" is shown in Supplementary Table S2. We hypothesized that *F. nucleatum* might contribute to the acquisition of aggressive tumor behavior through the activation of chemokines. CCL20 was identified as the most upregulated chemokine (Supplementary Table S2), and we therefore evaluated the expression status of CCL20 in esophageal

**Table 2.** Cox regression analyses for cancer-specific survival

Characteristics	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P	HR (95% CI)	P
Age (for 10-year increase)	1.71 (0.50–6.10)	0.39		
Male(vs. female)	1.55 (0.73–4.02)	0.27		
Tobacco use (yes vs. no)	1.09 (0.60–2.20)	0.79		
Alcohol use (yes vs. no)	1.12 (0.60–2.33)	0.74		
Performance status 1–2 (vs. 0)	2.27 (1.36–3.67)	0.0020	2.16 (1.20–3.85)	0.011
Comorbidity present (vs. absent)	1.09 (0.67–1.84)	0.72		
Upper tumor location (vs. lower)	1.05 (0.55–1.85)	0.87		
Preoperative therapy present (vs. absent)	3.58 (2.25–5.80)	<0.0001		
Tumor stage III–IV (vs. stage I, II)	4.48 (2.75–7.54)	<0.0001	2.83 (1.51–5.44)	0.0012
<i>Fusobacterium nucleatum</i> positive (vs. negative)	2.01 (1.22–3.23)	0.0068	1.78 (1.06–2.94)	0.032



**Figure 3.**

Relationship between *F. nucleatum* DNA status in esophageal cancer and cancer-specific survival. Log<sub>e</sub> (HRs) plots of cancer-specific survival rate in *F. nucleatum* DNA-positive and -negative groups are shown. PS, performance status.

cancer tissues by IHC (Fig. 4B). We confirmed that the presence or absence of *F. nucleatum* was significantly associated with CCL20 expression status (Fig. 4C).

## Discussion

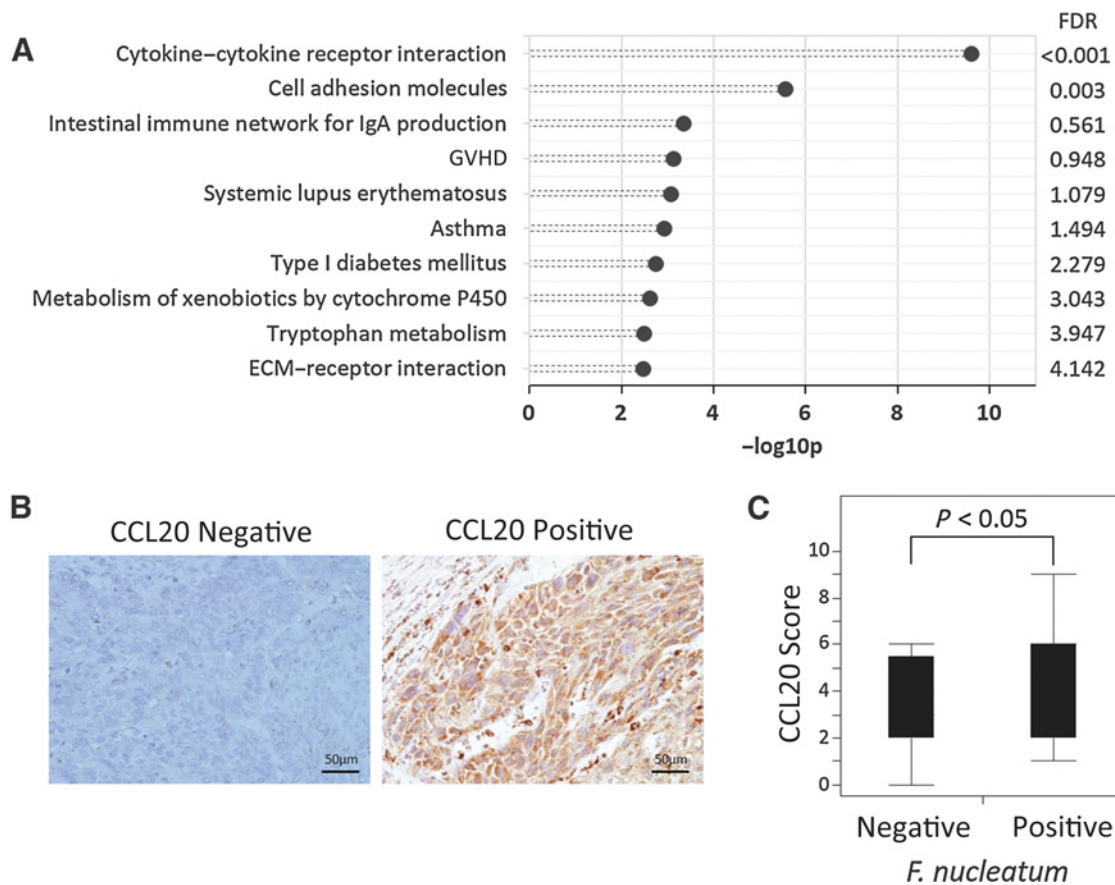
We examined the prognostic impact of human microbiome *F. nucleatum* among 325 patients with resected esophageal cancers. It is becoming increasingly clear that the human microbiome influences cancer development and progression (5, 30–32). A better understanding of the mechanisms and contribution of the microbiota to human cancers may thus aid the development of novel approaches to cancer treatment and/or prevention. To the best of our knowledge, the current study provides the first evidence for the relationship between *F. nucleatum* and poor prognosis in esophageal cancer. In addition, using KEGG enrichment analysis, we demonstrated that *F. nucleatum* might contribute to the acquisition of aggressive tumor behavior through the activation of chemokines, such as CCL20.

Previous studies of the relationships between *Fusobacterium* species and clinical outcome in human cancers have been inconclusive (Supplementary Table S1). Two studies reported that tumor *Fusobacterium* species were associated with a poor prognosis in patients with colorectal cancer (32, 33). Mima and collea-

gues analyzed a molecular pathology epidemiologic database of more than 1,000 colorectal cancers and revealed that *F. nucleatum* DNA levels in colorectal cancer tissues were associated with shorter survival (24), while Flanagan and colleagues demonstrated a relationship between *F. nucleatum* and poor prognosis in 122 colorectal cancers (23). Regarding pancreatic cancer, Mitsuhashi and colleagues reported that tumor *Fusobacterium* status was independently associated with a poorer prognosis (33). Our current findings in relation to esophageal cancer are in agreement with these previous results. In contrast, however, two other studies ( $n = 99$  and  $n = 511$ , respectively) of colorectal cancer found no associations between *Fusobacterium* detection and clinical outcome (20, 25). This discrepancy may be attributable to differences in patient cohorts or in the methods used to assess *Fusobacterium* species, or simply to chance variations between independent studies. However, our results demonstrated a clear association between increased *F. nucleatum* DNA content in esophageal cancer tissues and a poor prognosis, suggesting that *F. nucleatum* may be a suitable biomarker for identifying patients likely to experience inferior outcomes.

Experimental studies have provided mechanistic insights into the relationship between *F. nucleatum* and cancer progression. *F. nucleatum* is known to express the novel adhesin protein, FadA, on the bacterial cell surface (34). Rubinstein and colleagues revealed that FadA can bind to E-cadherin, activate  $\beta$ -catenin signaling, and promote colorectal cancer cell proliferation in *in vitro* and *in vivo* models (22). They also reported that colorectal cancer tissues showed elevated *fadA* gene levels, suggesting a potential role of FadA as a diagnostic and therapeutic target in human cancer. *F. nucleatum* was shown to inhibit T cell-mediated immune responses against colorectal tumors and promote tumor progression in the *Apc*<sup>Min/+</sup> mouse model (35). Another study using colorectal cancer samples revealed an inverse association between tissue levels of *F. nucleatum* DNA and CD3<sup>+</sup> T-cell density in tumor tissues (28). Collectively, these results suggest that *F. nucleatum* may exert immunosuppressive activity by inhibiting human T-cell responses. A third possible mechanism involves modulation of the tumor immune microenvironment. An *in vivo* study showed that colonization by *F. nucleatum* stimulated the secretion of immune cytokines, leading to colon tumorigenesis (35). Furthermore, our study revealed that the "cytokine–cytokine receptor interaction" was the most upregulated pathway in *F. nucleatum*-positive esophageal cancer, thereby supporting this third possible mechanism.

Accumulating evidence has demonstrated the crucial roles of chemokines and their receptors in tumor development and progression in several types of cancers (36–39). Kretschmer and colleagues recently reported that esophageal SCC cells modulated chemokine expression in fibroblasts, affecting the tumor immune response (40). The most upregulated chemokine in *F. nucleatum*-positive esophageal cancers in the current study was CCL20. An increasing number of studies have recently drawn attention to the roles of CCL20 and its physiologic sole receptor CCR6 in the development and progression of various type of cancers (41–43). An *in vitro* assay demonstrated that CCL20 stimulation promoted cancer cell proliferation and migration (44, 45). In addition, CCL20 plays crucial roles in the migration of Treg lymphocytes (46, 47), and the accumulation of Treg lymphocytes is associated with shorter survival in human cancers (48, 49). Liu and colleagues recently reported that CCL20 was related to tumor infiltration of Treg lymphocytes in esophageal SCC, suggesting the



**Figure 4.** *F. nucleatum* and chemokines in esophageal cancer. **A**, top 10 most enriched KEGG pathways among significantly upregulated DEGs in *F. nucleatum*-positive esophageal cancer tissues. Dashed lines, *P* values for the 10 top-ranked categories of KEGG pathways. The *P* values are expressed as the negative logarithm (base 10). **B**, immunostaining for CCL20 in esophageal cancer tissues. CCL20 positivity was observed in the cytoplasm and membrane of esophageal cancer cells. **C**, CCL20 expression scores were significantly higher in *F. nucleatum*-positive tissues (*n* = 20) compared with *F. nucleatum*-negative tissues (*n* = 20).

importance of chemokines, such as CCL20, in immune surveillance in esophageal cancer patients (50). Further studies are needed to validate the current findings and to elucidate the mechanism(s) whereby *F. nucleatum* affects tumor behavior.

In conclusion, *F. nucleatum* was detected in esophageal cancer tissues and was associated with shorter survival, suggesting that it may serve as a useful prognostic biomarker. *F. nucleatum* might also contribute to the acquisition of aggressive tumor behavior through the activation of chemokines, such as CCL20.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors' Contributions**

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**Writing, review, and/or revision of the manuscript:** K. Yamamura, Y. Baba, Y. Sakamoto, Y. Yamashita, M. Watanabe, H. Baba

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