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Intratumoral *Fusobacterium nucleatum* levels predict therapeutic response to neoadjuvant chemotherapy in esophageal squamous cell carcinoma

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Abstract

Purpose: Emerging evidence indicates that gut microbiome plays a crucial role in the cancer pathogenesis. Although *Fusobacterium nucleatum* (*F. nucleatum*) is associated with poor prognosis in multiple cancers, its clinical significance in predicting response to chemotherapy in patients with esophageal squamous cell carcinoma (ESCC) remains unclear.

Experimental design: The *F. nucleatum* levels were quantified by qPCR assays in tumor tissues from 551 ESCC patients from two independent cohorts, including 101 patients who received neoadjuvant chemotherapy prior to curative resection. Associations between *F. nucleatum* burden and recurrence free survival (RFS), as well with chemotherapeutic response were evaluated using response evaluation criteria in solid tumors (RECIST), primary tumor metabolic response defined by maximum standardized uptake value (SUVmax) changes in positron emission tomography - computed tomography (PET/CT), and pathological tumor regression grade (TRG).

Results: High burden of *F. nucleatum* in ESCC patients associated with poor RFS in both training (log-rank p=0.02; Hazard Ratio [HR]=1.61; p=0.03) and validation cohorts (log-rank p=0.003; HR=1.96; p=0.004). Importantly, ESCC patients with high levels of *F. nucleatum*

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displayed poor chemotherapeutic response for all three evaluation methods: RECIST (p=0.04), SUVmax change in PET/CT (p=0.0004), and TRG (p=0.003).

Conclusions: We conclude that high levels of intratumoral *F. nucleatum* have a prognostic significance for predicting poor RFS in ESCC patients. More importantly, our data indicates that higher *F. nucleatum* burden correlates with poor response to neoadjuvant chemotherapy, suggesting the possibility that an antibiotic intervention against this bacterium may significantly improve therapeutic response in ESCC patients.

Keywords

Fusobacterium nucleatum; esophageal cancer; prognosis; neoadjuvant chemotherapy; response

Introduction

Esophageal cancer is the sixth most common cause of cancer-related deaths worldwide (1). In spite of advances in multimodal therapies, including surgical removal of tumors, chemotherapy, radiotherapy and chemoradiotherapy, esophageal cancer remains a malignancy with high degree of fatality and overall 5-year survival rates of 15 to 20% (2, 3). Globally, esophageal squamous cell cancer (ESCC) is the predominant histological subtype of esophageal cancer (4). In particular, ESCC cases make up to 80% of all esophageal cancers in developing countries (5). The standard treatment strategy for locally advanced esophageal cancer in western and Asian countries comprise of neoadjuvant chemoradiotherapy or chemotherapy (NAC), followed by surgery (6, 7). Previous studies have shown that patients who respond well to NAC often exhibit improved overall survival (8, 9). A combination of cisplatin and 5-fluorouracil (5-FU) is currently used as standard chemotherapeutic regimen for esophageal cancer patients; however, the reported response rates remain relatively poor (10, 11). A recent study reported that addition of docetaxel to this regimen significantly improved the therapeutic response in patients with node-positive esophageal cancer (12). Nevertheless, most tumors acquire resistance to chemotherapeutic agents with subsequent treatment failure. Furthermore, there is currently no effective molecularly-targeted therapy available for esophageal cancer, and the efficacy of immunotherapy in these patients remains unclear.

In order to improve treatment response in esophageal cancer patients, it is of paramount importance to elucidate the underlying mechanism(s) that confer chemotherapeutic resistance in these patients. It has been postulated that cancer chemoresistance is attributed to complex interplay between gene regulation and external environmental factors. In this context, in recent years, gut microbiota has garnered a lot of attention in various malignancies, and it has been linked to both initiation and progression of gastrointestinal cancers through modulation of intestinal inflammation (13–15) and tumor-related signaling pathways (16). Recent studies have demonstrated that composition of the gut microbiome can significantly influence response to immunotherapy (17) and chemotherapy (18). Two recent independent studies identified an overabundance of *Fusobacterium nucleatum* (*F. nucleatum*) in colorectal cancer (CRC) tissues using metagenomic analysis (19, 20) and a high prevalence of *F. nucleatum* in these tissues associated with worse overall survival (21). In line with these observations, we identified that even in ESCC patients, the presence of

intratumoral *F. nucleatum* in neoplastic tissues was significantly associated with poor patient survival (22). Interestingly, building upon this growing evidence, a recent study reported that CRC patients who experienced increased incidence of tumor recurrence also possessed significantly higher burden of intratumoral *F. nucleatum* in their primary cancer tissues compared to those who did not exhibit tumor recurrence (23). In functional studies, *F. nucleatum* has been shown to enhance CRC chemoresistance through modulation of autophagy (23). In spite of the collective evidence highlighting the clinical importance of *F. nucleatum* in gastrointestinal cancers, whether changes in its expression levels contributes to patient prognosis and chemotherapeutic response in ESCC patients has not yet been explored.

Accordingly, in the present study we report that increased levels of intratumoral *F. nucleatum* associate with advanced tumor stage and poor survival. We also observed that higher burden of this microorganism in ESCC tissues predicted recurrence-free survival (RFS), as well as associated with poor response to NAC in patients with ESCC.

Materials and methods

Patients and sample collection procedures

This study analyzed a total of 551 cases with ESCC, which consisted of two independent clinical cohorts. A first patient cohort (training) included 207 ESCC patients, who were surgically treated at the Nagoya University Hospital, Nagoya, Japan, between October 2001 and October 2015. The second patient cohort (validation) comprised of 344 ESCC patients who underwent surgical resections, including 316 with radical surgeries, at the Kumamoto University Hospital, Kumamoto, Japan, between 2005 and 2016. Furthermore, this cohort included 187 patients that experienced surgery alone, 41 who received neoadjuvant chemoradiation therapy and 116 patients with NAC. Among these 116 patients in the NAC group, 101 patients were treated with two cycles of docetaxel, cisplatin and 5-FU (DCF) regimen. The study workflow is summarized in Supplementary Figure S1. Tumor depth (clinical T1–3) and regional lymph node involvement without distant metastases (N1) were used as the selection criteria for selecting patients for NAC treatment. Recurrence-free survival (RFS) was defined as the time period between the date of surgery to the time of tumor recurrence or death. Our study was conducted in accordance with the Declaration of Helsinki. A written informed consent was obtained from each patient, and the institutional review boards of all participating institutions approved this study. The patient characteristics are summarized in Table 1. The median follow-up duration for all cases after surgery was 20.4 months in the training cohort and 31.5 months in the validation cohort. The pathological diagnosis of all ESCC tumor tissue specimens was confirmed histologically, and the tumor node-metastasis (TNM) staging was determined according to the American Joint Committee on Cancer staging handbook (7th edition) (24), prior to and after surgery.

Patient treatments

The NAC regimen consisted of 2 hour intravenous administration of 60 mg/m^2 docetaxel beginning on day 1, a 24 hour continuous intravenous infusion of 350 mg/m^2 5-FU from days 1 through 5, and 1 hour intravenous administration of 6 mg/m^2 cisplatin from days 1

through 5. Two scheduled courses of NAC regimen were administered 3 weeks apart prior to esophagectomy. Surgery was carried out within 4 to 6 weeks following the final treatment day of preoperative chemotherapy, when curative resection was considered feasible.

DNA extraction and quantitative polymerase chain reaction (qPCR) assays

Genomic DNA from fresh frozen tissues in the training cohort were extracted using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany). Likewise, genomic DNA from the formalin-fixed paraffin-embedded (FFPE) tissues in the validation cohort were extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen). The amount of *F. nucleatum* DNA was quantified by use of a qPCR assay. The *nus G* gene of *F. nucleatum* and the reference human gene *SLCO2A1* were amplified using custom TaqMan primer/probe sets (Applied Biosystems, Carlsbad, CA, USA) in 384-well optical PCR plates, as described previously (22).

Evaluation of response to chemotherapy using RECIST

The response to chemotherapy was assessed in the validation cohort using response evaluation criteria in solid tumors (RECIST) Version 1.1 (25). Briefly, computed tomography (CT) images were analyzed using with the following definitions: complete response (CR), as disappearance of all clinical and radiological evidence of the tumor; partial response (PR), as decrease of 30% or more in the sum of longest diameters of all target measurable lesions; progressive disease (PD), as increase of more than 20% of the sum of longest diameters of all target measurable lesions or the appearance of new lesions; and stable disease (SD), as all other indications. Patients with CR or PR were defined as responders, while PD and SD were classified as non-responders.

PET/CT imaging

A total of 86 out of 101 patients who received NAC also underwent positron emission tomography - computed tomography (PET/CT) using a hybrid PET/CT imager, consisting of a dedicated GSO full-ring PET scanner and a 16-slice helical CT scanner (Gemini GXL16, Philips Medical Solutions, Amsterdam, Netherlands). All patients fasted for a minimum of 5 h prior to the examination. Emission scans were acquired in a 3D mode, with a 144×144 matrix, 60 min after intravenous injection of 185–300 MBq 18F-fluoro-deoxy-glucose (FDG), immediately after urination. PET/CT transmission data were acquired for the area defined from the base of the skull to the proximal thighs. Standardized uptake value (SUVmax) response was classified as follows (26): complete metabolic response (CMR), as complete resolution of FDG uptake within the measurable target lesion, with the appearance of no new lesion; partial metabolic response (PMR), with at least 30% reduction in SUVmax of FDG uptake; progressive metabolic disease (PMD), with more than 30% increase in the SUVmax of the FDG uptake or appearance of FDG avid new lesion/s that is/are morphologically typical of cancer; stable metabolic disease (SMD), as disease which did not qualify for CMR, PMR, or PMD. Patients with CMR or PMR were defined as responders.

Pathological tumor regression grading criteria

The histopathological response to NAC was classified into four categories according to the following criteria (27): grade 1, as no evidence of viable tumor cells; grade 2, with less than 10% viable tumor cells; grade 3, with 11–50% viable tumor cells; and grade 4, with more than 50% viable tumor cells. Subsequently, grade 1–3 tumors were combined as the group of patients with response (TRG 1–3), while grade 4 tumors were classified as non-responders (TRG 4).

Statistical analysis

All statistical analyses were carried out using JMP, version 10 (SAS Institute, Cary, NC, USA). Continuous variables were expressed as medians and were compared using a t-test or Mann–Whitney U test. Categorical variables were compared using chi-squared or Fisher's exact test. All p-values were calculated using a two-sided test, and a p-value of <0.05 was considered statistically significant. For time-to-event analyses, survival estimates were calculated using the Kaplan-Meier analysis, and the survival differences between groups were compared using the log-rank test. Associations between RFS and clinicopathological features was evaluated by univariate Cox proportional hazards regression analysis. Parameters determined to be significant by univariate analysis were included in multivariate Cox proportional hazards regression analysis. Similarly, we analyzed associations between chemotherapeutic response and clinicopathological features using by univariate and multivariate logistic regression analysis.

Results

The levels of *F. nucleatum* are significantly higher in ESCC patients

We first assessed the burden of *F. nucleatum* in ESCC tissues by a qPCR assay in two independent patient cohorts, where matched cancer and normal tissues were available. We observed that *F. nucleatum* DNA levels were significantly higher in cancer tissues compared to the paired adjacent normal tissues in both cohorts (training cohort; n=45, P=0.006; validation cohort; n = 48, P <0.0001; Figure 1A and 1B, respectively).

We next analyzed the abundance of *F. nucleatum* in the training (n=207) and validation (n=344) cohorts, based upon all tumor stages. Interestingly, we observed a marked enrichment of *F. nucleatum* in ESCC patients with advanced (T2-T4) vs. those with an earlier stage disease (T1), in both cohorts (P <0.05; Figure 1C and 1D).

Higher levels of *F. nucleatum* associated with advanced stage disease in ESCC

Next, we determined the associations between *F. nucleatum* burden and various clinicopathological features in two independent ESCC patient cohorts (training cohort; n=207 and validation cohort; n=344). The cut-off thresholds to categorize tumors into the high and low groups were determined using ROC analysis and Youden's index, based on the level of *F. nucleatum* that provided the highest sensitivity and specificity to predict ESCC recurrence in the training cohort. The same cut-off values were then applied to the patient in the validation cohort to evaluate survival. We observed that there was no effect of age (p=0.26), gender (p=0.62), tumor location (p=0.29), or N stage (p=0.88) on the expression of

expression of *F. nucleatum* in the cancer tissues within the training cohort. Similar results were noted in the validation cohort for age (p=0.82), gender (p>0.99), location (p=0.42), and N stage (p=0.12).

However, in the training cohort, high intratumoral *F. nucleatum* levels were associated with higher T category (p=0.03) and in patients who received preoperative treatment (p=0.03). Similarly, high levels of intratumoral *F. nucleatum* were significantly associated with larger tumor size (p=0.004), higher T category (p<0.001), higher TNM stage (p=0.03), and in patients who underwent preoperative treatment (p=0.01) in the validation cohort (Table 1). Collectively, our results indicate that high levels of *F. nucleatum* associate with an invasion depth in ESCC patients.

Increased burden of *F. nucleatum* associate with higher tumor recurrence, poor RFS, and serve as a prognostic indicator for early stage ESCC patients

Considering that presence of *F. nucleatum* in cancer cells is associated with advanced disease, we were curious to interrogate its relationship with tumor recurrence in ESCC patients. Therefore, we determined the relationship between the *F. nucleatum* levels and cancer recurrence in the training cohort of 207 patients (87 patients with recurrence and 120 without recurrence), wherein we observed a significant association for higher *F. nucleatum* levels in patients with recurrence (P=0.04; Figure 2A). Likewise, these findings were subsequently confirmed in the validation cohort of 316 ESCC patients, which included 91 patients without recurrence and 225 with recurrence. Here again, we noted that the overall levels of *F. nucleatum* were significantly higher in neoplastic tissues in ESCC patients with recurrence vs. those without recurrence (P=0.01; Figure 2B).

In order to determine whether intratumoral *F. nucleatum* burden in ESCC patients is associated with RFS, we performed Kaplan–Meier analysis in both cohorts. Interestingly, patients in the training cohort with high vs. low intratumoral *F. nucleatum* levels exhibited a significantly poor RFS (log-rank p = 0.02, Figure 2C); a finding which was also true when interrogated in the independent validation cohort (log-rank p = 0.003, Figure 2D).

Since we observed a higher burden of *F. nucleatum* in advanced ESCC patients (T2–4 vs. T1), we investigated whether the presence of this bacterium had any effect on patient survival, even in early ESCC. Advanced ESCC patients stratified by the T category alone (T2–4 vs. T1) exhibited poor prognosis in both patient cohorts (Supplementary Figure S2A and S2B). Importantly, however, when the T category was combined together with the *F. nucleatum* levels, we observed that even early stage T1 ESCC patients with high levels of this bacterium exhibited a worse RFS, which was similar to the one noted for patients with advanced disease, in both cohorts (training cohort: log-rank p = 0.002 and validation cohort: log-rank p = 0.009, Figure 2E and 2F, respectively). These findings highlight that presence of high levels of *F. nucleatum* indicate an important prognostic biomarker potential for this bacterium in ESCC patients.

High levels of *F. nucleatum* serve as an independent risk factor for RFS in ESCC patients

Next, we were curious to investigate the clinical significance of *F. nucleatum* levels in term of patient survival in the context of other clinicopathological features, using univariate and

multivariate analysis, in both patient cohorts. In the training cohort, univariate Cox regression analysis revealed that patients with proximal location of tumors (HR=2.04; 95% CI, 1.10–3.50; p= 0.02), and those with higher TNM stages (III/IV vs. I/II; HR=3.32; 95% CI, 2.05–5.63; p<0.0001), and those with high levels of *F. nucleatum* were associated with poor RFS (HR=1.61; 95% CI, 1.06–2.52; p=0.03; Table 2). These findings were further evaluated in a multivariate Cox model adjusted for various clinicopathological features, which were in agreement with our univariate analysis and demonstrated that proximal location of tumors (HR=3.09; 95% CI, 1.64–5.45; p=0.001), and those with higher TNM stages (III/IV vs. I/II; HR=3.78; 95% CI, 2.30–6.46; p<0.0001), and those with high levels of *F. nucleatum* were associated with poor RFS (HR=1.72; 95% CI, 1.12–2.70; p=0.01), suggesting that this bacterium was indeed an independent risk factor for predicting poor RFS in the patients within the training cohort.

We subsequently confirmed our findings in an independent validation cohort, wherein, once again we observed that in univariate analysis, preoperative therapy (HR=1.96; 95% CI, 1.29–3.00; p=0.002), TNM stages (HR=3.08; 95% CI, 2.03–4.72; p<0.0001), and higher burden of *F. nucleatum* (HR=1.96; 95% CI, 1.23–3.04; p=0.004) was significantly associated with worse RFS. Similarly, in multivariate analysis, TNM stages (HR=3.21; 95% CI, 1.81–5.70; p<0.0001) and high levels of *F. nucleatum* (HR=1.70; 95% CI, 1.06–2.65; p=0.03) were significantly associated with poor RFS. Collectively, these data demonstrate that high levels of intratumoral *F. nucleatum* are an independent risk factor for poor RFS in ESCC patients.

Intratumoral *F. nucleatum* burden correlates with worse chemotherapeutic response in ESCC patients

We examined whether higher burden of intratumoral *F. nucleatum* have any correlation with response to NAC in ESCC patients. We first investigated this association in the context of imaging data available to us from the CT scans. Of the 101 patients who underwent NAC treatment in the validation cohort, the *F. nucleatum*-high group had a significantly lower number of responders (i.e. patients with CR or PR; 42.9% (12/28) vs. 67.1% (49/73) in the *F. nucleatum*-low group; p = 0.04; Figure 3A and 3B).

Next, we interrogated this correlation as determined by the metabolic response rates determined by SUVmax values obtained from PET/CT imaging. Reassuringly, these analyses also revealed that patients with higher burden of *F. nucleatum* had a significantly fewer responders (i.e. patients with CMR or PMR; 47.6% (10/21) vs. 87.7% (57/65) in the low *F. nucleatum* group; p = 0.0004; Figure 3C and 3D).

Finally, we performed the pathological assessment of all patients based upon tumor regression grade (TRG) analysis. In these analyses, we noted that *F. nucleatum* levels were significantly higher in ESCC patients with a low vs. high pathological response (TRG 4 vs. TRG 1, 2 and 3; p = 0.003; Figure 3E and 3F). Taken together, these results illustrate that patients with high intratumoral levels of *F. nucleatum* appear to have greater resistance to NAC treatment.

High levels of *F. nucleatum* serve as an independent risk factor for predicting response to neoadjuvant chemotherapy in ESCC patients

Next, we analyzed the results of CT (RECIST), PET/CT and TRG in univariate and multivariate settings to determine the clinical significance of *F. nucleatum* as a potential biomarker of chemotherapeutic response in ESCC patients belonging to the validation cohort. The univariate logistic regression analysis revealed that higher levels of *F. nucleatum* associated with an overall poor chemotherapeutic response to NAC in all three approaches (RECIST: Odds Ratio [OR], 2.72; 95% CI 1.12–6.78, p = 0.03; PET/CT: OR, 7.84; 95% CI 2.58–25.4, p = 0.0003; and TRG: OR, 11.6; 95% CI 2.25–214, p = 0.001; Table 3).

Likewise, multivariate analysis also revealed that high levels of intratumoral *F. nucleatum* burden was an independent risk factor for poor response to NAC in all three criteria (RECIST: OR, 2.97; 95% CI 1.19–7.73, p = 0.02; PET/CT: OR, 7.66, 95% CI 2.37–26.8, p = 0.0006; and TRG: OR, 10.3; 95% CI 1.96–190, p = 0.003). Collectively, these results illustrate that *F. nucleatum* is an important independent risk factor and a potential biomarker for predicting response to NAC in ESCC patients.

Discussion

With the growing recognition for the role of microbiome in human disease, over the last decade, one such organism, *F. nucleatum*, has been identified as an important bacterium linked to the pathogenesis of multiple human cancers. In this present study, we for the first time, interrogated the clinical significance of *F. nucleatum* as a potential prognostic and predictive biomarker of response to neoadjuvant chemotherapy in large, multiple, independent cohort of ESCC patients, and for the first time, investigated *F. nucleatum* as a potential predictive biomarker of response to neoadjuvant chemotherapy. In this study, we make several novel observations. First, we demonstrate that *F. nucleatum* burden is significantly higher in ESCC patients with advanced disease stage. Second, we describe that higher levels of this bacterium are present in patients with recurrence, and are an independent risk factor for predicting poor RFS in ESCC. Third, we illustrate that using RECIST, PET/CT and TRG analysis, higher burden of *F. nucleatum* predicts poor response to neoadjuvant chemotherapy (NAC) in ESCC patients; collectively highlighting the potential possibility of its prognostic and predictive biomarker utility, as well as suggest the possibility of using an antibiotic intervention to target this bacterium for improving the therapeutic response rates to chemotherapy in ESCC patients. In addition as we developed a PCR-based cut-off to measure the *F. nucleatum* levels in the training cohort patients, and subsequently applied these in an independent validation cohort, we are enthused that our findings can be further validated in a prospective settings for response prediction to neoadjuvant chemotherapy.

It has been recognized that *F. nucleatum* is frequently present in the human oral cavity, and acts as a pathogen in periodontal disease (28). Recently, several studies have reported that high *F. nucleatum* burden correlates with poor prognosis in colorectal cancer (21, 29, 30). Moreover, we previously reported a similar positive correlation between high *F. nucleatum* levels and poor overall and cancer specific survival in ESCC patients (22). The potential role of *F. nucleatum* in gastrointestinal cancers is poorly understood. Experimental evidence in

colorectal cancer has provided mechanistic insights that *F. nucleatum* expresses adhesin protein, FadA, on the bacterial cell surface. FadA can bind to E-cadherin, activates b-catenin signaling, and promotes colorectal cancer cell proliferation (31). In this study, *F. nucleatum* burden was significantly higher in ESCC patients with advanced stage. However, T1 ESCC patients with high levels of *F. nucleatum* exhibited a worse RFS, analogous to patients with advanced disease, suggesting that this bacterium, even in early stage ESCC patients promotes aggressive tumor behavior and could impact patient prognosis.

To further investigate the role of *F. nucleatum*, it was recently demonstrated that mice bearing colorectal cancer and treated with an antibiotic were found to have lower levels of this baterium and exhibited reduced cell proliferation and tumor growth, suggesting that antibiotics may be helpful in the treatment of *F. nucleatum*-associated cancers (32). Accumulating evidence suggests that the gut microbiota modulates local immune response, and in turn might alter the efficacy of chemotherapy (18, 33) and immunotherapy (34, 35). In one such study, chemotherapeutic response was modulated by adaptive immunity in ovarian cancer (36). Although these preclinical evidences indicates that microbiota appears to modulate chemotherapeutic response in multiple cancer types (37, 38), to the best of our knowledge, none of the studies have thus far evaluated the clinical significance of *F. nucleatum* in the context of responsiveness to chemotherapeutic treatment in cancer patients. Herein, we fill this important gap in knowledge, and evaluated therapeutic response using three commonly used and well-established approaches for drug resistance in ESCC patients. In spite of the use of RECIST as one of the most widely used tumor response metric (25), it has several limitations due to its dependence on morphologic changes (39). RECIST criteria can often select lymph nodes as target lesions in ESCC patients. In contrast, 18F-FDG PET is considered as a superior method which overcomes the limitations of RECIST. Since metabolic changes are thought to be more closely related to malignant potential of tumors (40), PET/CT is emerging as a more accurate non-invasive imaging modality for initial staging and response assessment in ESCC patients (41). Based on these findings, PET response criteria in solid tumors (PERCIST), which is RECIST using 18F-FDG PET, has recently been proposed as an optimal method for standardized evaluation of the metabolic tumor response rates (39).

In the present study, we observed significant differences between response classifications and *F. nucleatum* levels in ESCC tissues. Interestingly, PET response and tumor regression grade (TRG) were more strongly associated with *F. nucleatum* levels compared to RECIST, in ESCC patients receiving NAC treatment. While RECIST in ESCC patients primarily evaluates shrinkage of lymph nodes, PET/CT and TRG reflect the response of the primary tumor itself. In this study, since *F. nucleatum* levels in tumor tissues correlate with higher T category, our data imply that this bacterium might be involved in modulating chemotherapeutic response more directly. While specific mechanism(s) underlying chemotherapeutic response of *F. nucleatum* in cancer remain unclear, several studies have investigated bacteria-induced drug resistance using *in vitro* and *in vivo* models. Yu et al. reported that *F. nucleatum* activates autophagy-related pathways in colorectal cancer through modulation of TLR4 and MYD88 innate signaling, along with certain miRNAs which subsequently promote chemoresistance (23). Likewise, Geller and colleagues reported that intratumoral gamma-proteobacteria modulated the chemotherapeutic response by converting

gemcitabine into an inactive metabolite through regulation of cytidine deaminase in pancreatic cancer (42). Nonetheless, further studies are required to interrogate and validate the findings of the current study, and elucidate the mechanisms by which *F. nucleatum* modulates the chemotherapeutic response in ESCC patients.

Although our results indicate that *F. nucleatum* levels could serve as a potential biomarker for predicting survival and response to NAC in ESCC patients, there are certain limitations of our study. The detection rates of *F. nucleatum* were different between the training and validation cohorts. We analyzed frozen tissues in the training cohort of patients, and FFPE tissues in the validation cohort. The qPCR method is currently the most commonly used method for the quantification of *F. nucleatum* levels; however, most the detection rates of *F. nucleatum* in frozen tissues are generally higher vs. FFPE tissues (43, 44). There is a possibility that tissue fixation during the processing of FFPE tissues might be an important factor for the reduced detection rates of *F. nucleatum* (45) in clinical specimens.

In conclusion, we demonstrate that high intratumoral *F. nucleatum* levels associated with tumor recurrence and poor RFS in two large, independent cohorts of ESCC patients. More importantly, our results indicate that high burden of *F. nucleatum* in ESCC is predictive of response to neoadjuvant chemotherapy. Collectively, our data highlight that *F. nucleatum* is not only an important predictive biomarker of chemotherapeutic response, but might be a potential target of antibiotic intervention for improving the therapeutic response rates in ESCC patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational relevance

Esophageal squamous cell carcinoma (ESCC) is a disease with high mortality rates. The standard treatment strategy for locally advanced ESCC comprises of neoadjuvant chemoradiotherapy or chemotherapy (NAC), followed by surgery; however, there is lack of availability of adequate biomarkers to predict chemotherapeutic response. *Fusobacterium nucleatum* (*F. nucleatum*) has been identified as an important bacterium linked to the pathogenesis of multiple human cancers. In this study, we investigated its clinical significance in ESCC patients and demonstrated that higher levels of *F. nucleatum* are an independent risk factor for predicting poor recurrence free survival. Furthermore, we illustrate that using RECIST, PET/CT and TRG analysis, higher burden of *F. nucleatum* predicts poor response to NAC. Our data highlight that *F. nucleatum* levels serve as an important prognostic and predictive biomarker, and suggest the possibility of using an antibiotic intervention to target this bacterium for improving the chemotherapeutic response in ESCC patients.

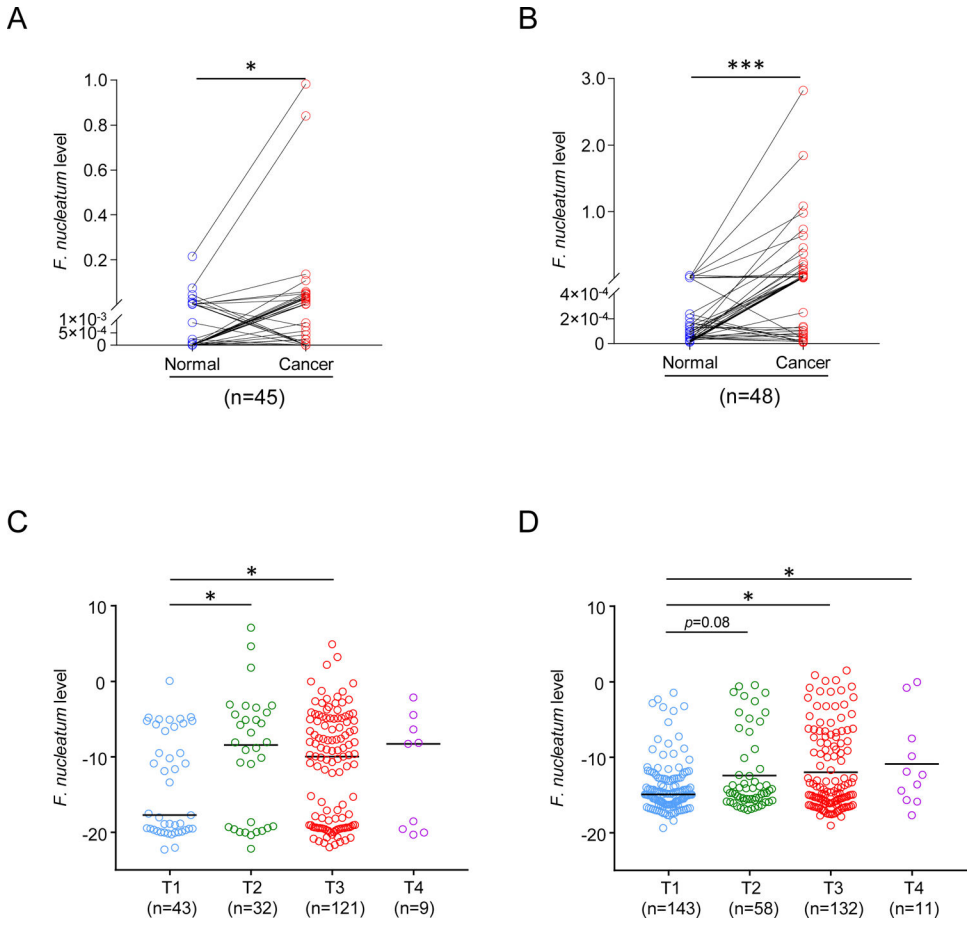

Figure 1: *F. nucleatum* expression in patients with ESCC.

(A) The expression of *F. nucleatum* in 45 pairs of ESCC and adjacent normal tissue in the training cohort, and (B) in 48 pairs of the validation cohort. (C) The relative amount of *F. nucleatum* in 207 ESCC tissue according to T category in training cohort, and (D) in 344 ESCC tissue in validation cohort. *p<0.05; **p<0.01, ***p<0.001

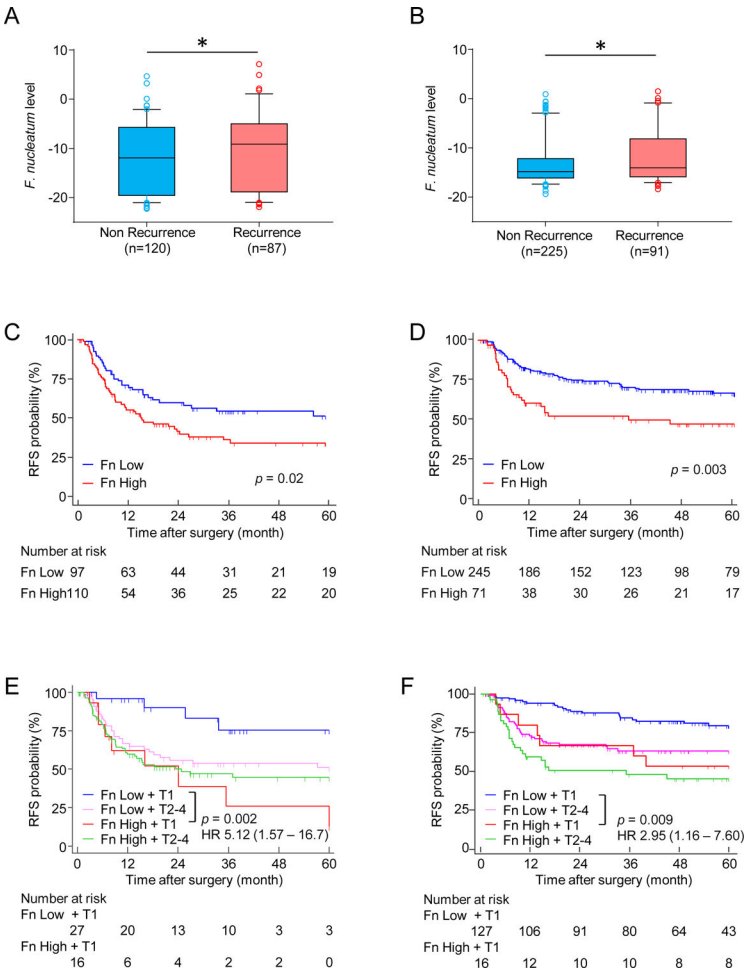

Figure 2: High intratumoral *F. nucleatum* is associated with worse prognosis in ESCC.

Comparison of *F. nucleatum* expression levels in patients with or without recurrence in (A) the training and (B) the validation cohort. Kaplan-Meier analysis of RFS for ESCC patients with high (red) or low (blue) *F. nucleatum* levels in (C) the training and (D) the validation cohort. Kaplan-Meier analysis of RFS for ESCC patients with low *F. nucleatum* levels in T1 (blue) or T2–4 tumor (pink), or high *F. nucleatum* levels in T1 (red) or T2–4 tumor (green) in (E) the training and (F) the validation cohort. Fn indicates *Fusobacterium nucleatum*. *p<0.05

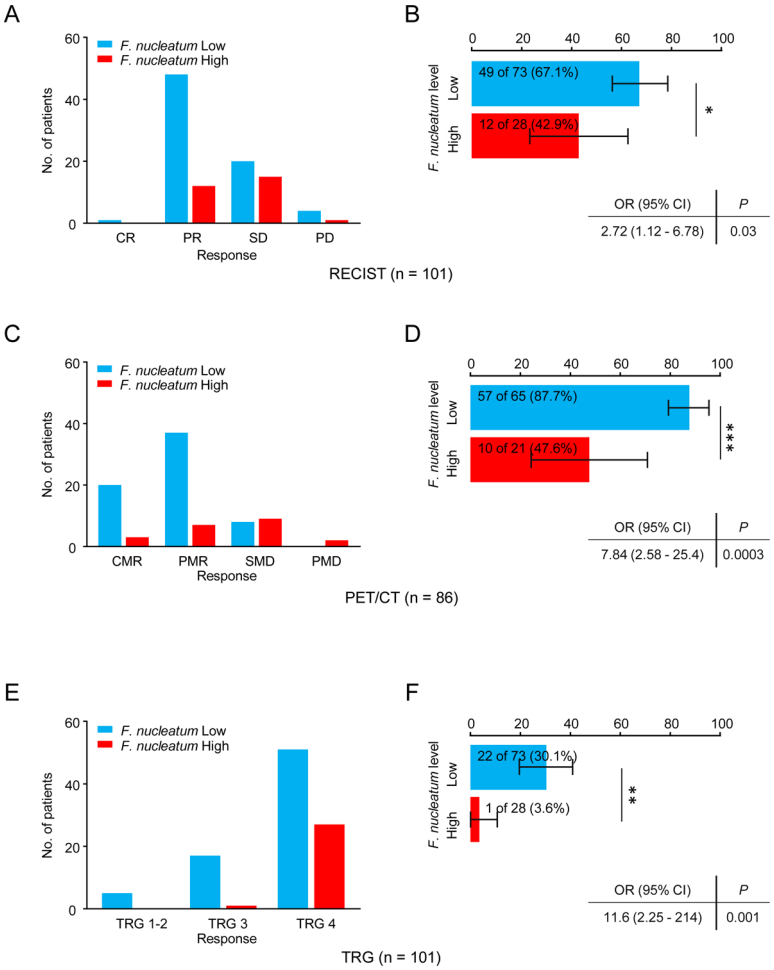

Figure 3: Intratumoral *F. nucleatum* levels are associated with chemotherapeutic response.

Chemotherapeutic response and the rate of responders in the validation cohort by comparing *F. nucleatum* high (red) and low (blue) patients using RECIST (A and B), PET/CT (C and D) and TRG (E and F). RECIST, response evaluation criteria in solid tumors. PET/CT, Positron Emission Tomography - Computed Tomography. TRG, Tumor Regression Grade. *p<0.05; **p<0.01, ***p<0.001

Table 1:

F. nucleatum expression levels and relationship with various clinicopathological features in ESCC patients

	Training cohort (n=207)			Validation cohort (n = 344)		
	***F. nucleatum* expression**			***F. nucleatum* expression**		
	Low	**High**	***P***	**Low**	**High**	***P***
Age (range)	66 (44–84)	66 (44–83)	0.26	66 (41–86)	65 (49–89)	0.82
Sex			0.62			> 0.99
Male	76	83		228	72	
Female	21	27		34	10	
Location			0.29			0.42
Upper	14	10		171	50	
Lower	83	100		91	32	
Tumor size, cm	4.5(1.5 – 17.0)	4.5 (2.2 – 14.0)	0.78	3.5 (1.1 – 15.0)	4.2 (1.2 – 14.5)	**0.004**
SCC, ng/mL	1.2(0.2 – 22.8)	1.2 (0.2 – 7.3)	0.44	NA	NA	NA
T category			**0.03**			**<0.0001**
T1	26	17		126	17	
T2 – 4	69	93		136	65	
Undefined	2	0		NA	NA	
Lymph node metastasis			0.88			0.12
Absent	34	38		141	33	
Present	63	72		121	49	
Tumor Stage			> 0.99			**0.03**
I – II	42	48		169	40	
III – IV	53	62		93	42	
Undefined	2	0		NA	NA	
Differentiation			0.16			NA
well-mod	79	93		NA	NA	
poor-	17	13		NA	NA	
Undefined	1	4		NA	NA	
Preoperative treatment			**0.03**			**0.01**
Present	41	59		108	49	

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	Training cohort (n=207)			Validation cohort (n = 344)		
	F. nucleatum expression			*F. nucleatum* expression		
	Low	High	*P*	Low	High	*P*
Absent	58	49		154	33	

Table 2:

High levels of *F. nucleatum* serve as an independent risk factor for predicting RFS in SESCC patients

	Training cohort (n=207)				Validation cohort (n=316)			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Age (< 65)	0.71 (0.91 – 2.21)	0.12			1.00 (0.66 – 1.53)	0.99		
Male	1.42 (0.85 – 2.49)	0.18			1.10 (0.60 – 2.25)	0.78		
Upper tumor	2.04 (1.10 – 3.50)	**0.02**	3.09 (1.64 – 5.45)	**0.001**	0.95 (0.62 – 1.48)	0.81		
Preoperative therapy	1.09 (0.71 – 1.66)	0.7			1.96 (1.29 – 3.00)	**0.002**	0.87 (0.49 – 1.53)	0.61
TNM stage (III–IV)	3.32 (2.05 – 5.63)	**<0.0001**	3.78 (2.30 – 6.46)	**<0.0001**	3.08 (2.03 – 4.72)	**<0.0001**	3.21 (1.81 – 5.70)	**<0.0001**
F. nucleatum High	1.61 (1.06 – 2.52)	**0.03**	1.72 (1.12 – 2.70)	**0.01**	1.96 (1.23 – 3.04)	**0.004**	1.70 (1.06 – 2.65)	**0.03**

Table 3:

Intratumoral *F. nucleatum* burden correlates with worse chemotherapeutic response in ESCC patients

Characteristics	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*	OR (95% CI)	*P*
RECIST				
Age (vs >= 65)	1.47 (0.65 – 3.41)	0.35		
Male (vs Female)	2.42 (0.68 – 11.3)	0.18		
Upper tumor location (vs lower)	1.14 (0.48 – 2.74)	0.77		
T category, 3–4 (vs 1–2)	0.83 (0.34 – 2.03)	0.68		
Lymph node metastasis	5.88 (1.02 – 111)	**0.04**	6.95 (1.19 – 135)	**0.03**
F. nucleatum High (vs Low *)*	2.72 (1.12 – 6.78)	**0.03**	2.97 (1.19 – 7.73)	**0.02**
PET/CT				
Age (vs >= 65)	1.65 (0.57 – 5.18)	0.35		
Male (vs Female)	2.10 (0.34 – 40.6)	0.47		
Upper tumor location (vs lower)	0.92 (0.32 – 2.93)	0.89		
T category, 3–4 (vs 1–2)	10.0 (1.89 – 186)	**0.004**	9.74 (1.66 – 187)	**0.008**
Lymph node metastasis	0.43 (0.09 – 2.27)	0.3		
F. nucleatum High (vs Low)	7.84 (2.58 – 25.4)	**0.0003**	7.66 (2.37 – 26.8)	**0.0006**
TRG				
Age (vs >= 65)	0.56 (0.20 – 1.49)	0.25		
Male (vs Female)	0.25 (0.01 – 1.38)	0.13		
Upper tumor location (vs lower)	1.53 (0.57 – 4.02)	0.39		
T category, 3–4 (vs 1–2)	2.56 (0.95 – 6.85)	0.06	2.05 (0.74 – 5.70)	0.17
Lymph node metastasis	1.88 (0.36 – 7.48)	0.45		
F. nucleatum High (vs Low)	11.6 (2.25 – 214)	**0.001**	10.3 (1.96 – 190)	**0.003**