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ORIGINAL ARTICLE

Relationship between gut microbiome *Fusobacterium nucleatum* and LINE-1 methylation level in esophageal cancer

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Abstract

Background We previously demonstrated the relationship of human microbiome *Fusobacterium nucleatum* with unfavorable clinical outcomes and inferior chemotherapeutic responses in esophageal cancer. Global DNA methylation is associated with the occurrence and development of various cancers. In our previous study, LINE-1 hypomethylation (i.e., global DNA hypomethylation) was associated with a poor prognosis in esophageal cancer. As the gut microbiota may play crucial roles in the DNA methylation of host cells, we hypothesized that *F. nucleatum* might influence LINE-1 methylation levels in esophageal cancer.

Methods We qualified the *F. nucleatum* DNA using a quantitative PCR assay and LINE-1 methylation via a pyrosequencing assay using formalin-fixed paraffin-embedded specimens from 306 esophageal cancer patients.

Results Intratumoral *F. nucleatum* DNA was detected in 65 cases (21.2%). The LINE-1 methylation scores ranged from 26.9 to 91.8 (median = 64.8) in tumors. *F. nucleatum* DNA was related to the LINE-1 hypomethylation of tumor lesions in esophageal cancer ($P < 0.0001$). The receiver operating characteristic curve analysis showed that the area under the curve was 0.71 for *F. nucleatum* positivity. Finally, we found that the impact of *F. nucleatum* on clinical outcomes was not modified by LINE-1 hypomethylation (P for interaction = 0.34).

Conclusions *F. nucleatum* alters genome-wide methylation levels in cancer cells, which may be one of the mechanisms by which *F. nucleatum* affects the malignant behavior of esophageal cancer.

Keywords Esophageal cancer · Biomarkers · Cancer epigenetics

Introduction

Esophageal cancer is one of the leading causes of cancer-related death worldwide. Despite the development of multidisciplinary treatments, including surgery, immunotherapy, chemotherapy, radiotherapy, and chemoradiotherapy, the prognosis of patients with esophageal cancer remains unfavorable [1]. The limited improvement in treatment outcomes with these conventional therapies has prompted the search for innovative therapeutic strategies [2, 3]. Importantly, epigenetic changes and alterations in the gut microbiome are reversible and can be targets for cancer therapy or chemoprevention [4–6].

Gut microbiota is a highly advanced research field that has attracted much attention in recent years because of its reported association with various diseases, such as obesity, inflammatory bowel disease and cancers. *Fusobacterium nucleatum* (*F. nucleatum*) is a gram-negative non-spore-specific anaerobic bacterium and a constituent of the oral microbiomes [7]. It can adhere to the oral cavity as well as migrate to and colonize the intestinal tract, which is closely related to the occurrence and development of various types of cancers, including esophageal cancer [8, 9]. *F. nucleatum* can activate the host cell cancer-related signal pathway and promote

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the proliferation and metastasis of cancer cells [10–12]. We have previously reported that the high amount of *F. nucleatum* DNA is related to a poor prognosis in patients with esophageal cancer [13]. In addition, *F. nucleatum* confers chemoresistance to esophageal cancer cells via the modulation of autophagy. However, the mechanism by which *F. nucleatum* contributes to esophageal cancer malignancy is not yet completely clear.

DNA methylation changes associated with human tumors are site-specific CpG island promoter hypermethylation and global DNA hypomethylation. Promoter hypermethylation can silence tumor suppressor genes, DNA mismatch repair genes (e.g., *MLH1*), or DNA repair genes (e.g., *MGMT*), thereby contributing to esophageal carcinogenesis [14]. Global DNA hypomethylation appears to play an important role in genomic instability, leading to cancer development [5]. As long interspersed element-1 (LINE-1 or L1; a repetitive DNA retrotransposon) constitutes approximately 17% of the human genome, the level of LINE-1 methylation is regarded as a surrogate marker of global DNA methylation [5]. LINE-1 methylation is highly variable, and the strong relationships between LINE-1 hypomethylation and unfavorable prognosis have been shown in many types of human cancers, such as esophageal cancer [15–18].

The gut microbiome can alter DNA methylation in host cells through a variety of mechanisms. Therefore, we hypothesized that the level of LINE-1 methylation in esophageal cancer tissues might be influenced by *F. nucleatum*. To our knowledge, this is the first study to focus on the relationship between *F. nucleatum* and LINE-1 methylation in esophageal cancer. This study provides new insights into the correlation between the amount of *F. nucleatum* DNA and the methylation level of LINE-1. Therefore, we inferred that the enrichment of *F. nucleatum* is associated with host tumor epigenetic modification in esophageal cancer.

Materials and methods

Study cohort

We analyzed 306 formalin-fixed paraffin-embedded (FFPE) specimens of esophageal cancer tissues from patients who underwent resection. The TNM stage was determined according to the American Joint Committee on Cancer Staging Manual (7th edition) [19]. We interviewed patients at their first visit for information about their smoking and drinking history and assessed whether they had ever smoked or drank alcohol (Yes or No). Table 1 shows the clinical features of the study cohort. Written informed consent was obtained from each patient, and the procedures were approved by the Institutional Review Board of Kumamoto University (#1272).

Quantitative real-time polymerase chain reaction (qPCR) for *F. nucleatum*

H&E-stained slides of the tumors were reviewed by one pathologist, who marked the areas of the tumor and normal mucosa. H&E-stained tissue sections from each case were scraped off slides for DNA extraction. The DNA was extracted using a QIAamp DNA FFPE tissue kit (Qiagen, Valencia, CA, USA). Whole genome amplification of genomic DNA was performed by PCR using random 15-mer primers for subsequent genetic analyses. 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, Waltham, Massachusetts, USA). Assays were performed in a 384-well optical PCR plate. DNA was amplified and detected using a LightCycler® 480 Instrument II (Roche, Basel, Switzerland). The amount of *F. nucleatum* DNA in each tissue was normalized relative to *SLCO2A1* [13]. In this study, 0 (i.e., cases with no *F. nucleatum* DNA detected at all) are classified as negative and > 0 (i.e., cases with even a little *F. nucleatum* DNA detected) are classified as positive.

Pyrosequencing for LINE-1 methylation

Bisulfite treatment of genomic DNA was carried out using an EpiTect Bisulfite kit. PCR and subsequent LINE-1 pyrosequencing were performed using a PyroMark Kit (Qiagen) [20]. This assay amplifies a region of LINE-1 element (position 305–331 in accession No. X58075), which includes four CpG cites. The PCR condition was 45 cycles of 95 °C for 20 s, 50 °C for 20 s and 72 °C for 20 s, followed by 72 °C for 5 min. The biotinylated PCR product was purified and made single-stranded to act as a template in a pyrosequencing reaction, using the Pyrosequencing Vacuum Prep Tool (Qiagen). Pyrosequencing reactions were performed in the PyroMark Q24 System (Qiagen). The nucleotide dispensation order was: ACT CAG TGT GTC AGT CAG TTA GTC TG. The amount of C relative to the sum of the amounts of C and T at each CpG site was calculated as percentage (i.e., 0–100). The average of the relative amounts of C in the four CpG sites was used as overall LINE-1 methylation level in a given tumor (Fig. 1).

Pyrosequencing to measure promoter methylation of MGMT and MLH1

Pyrosequencing for *MGMT* and *MLH1* was performed using the PyroMark kit (Qiagen) [21]. For each sample, the average of the four and five CpG islands were calculated,

Table 1 Clinical and pathological features of 306 cases

Clinical and pathological feature	*F.nucleatum*		*P* value
	Negative ($n=241$)	Positive ($n=65$)	
Mean age ± SD	65.6 ± 8.7	66.0 ± 9.5	0.65
Gender			0.27
Male	211	60	
Female	30	5	
Performance status			0.49
0	186	51	
1	47	10	
2	8	4	
Tobacco use			0.047
Yes	207	58	
No	34	7	
Alcohol use			0.34
Yes	230	60	
No	11	5	
Histology			0.61
Squamous cell carcinoma	222	62	
Adenocarcinoma	11	2	
Others	8	1	
Stage			0.0023
0	38	3	
I	64	8	
II	61	27	
III	56	19	
IV	22	8	
Preoperative therapy			0.069
Present	78	29	
Absent	163	36	

respectively. 182 cases could be analyzed for *MGMT* and *MLH1* methylation, because sufficient amounts of biotinylated PCR product were available (supplemental table). For *MGMT*, a clear pyrogram was not obtained in one case.

Statistical analysis

All statistical analyses were performed using JMP, version 10 (SAS Institute, Cary, NC, USA). All *P* values were two sided. Fisher's exact test and Student's *t* test were utilized to compare mean values for all variables. The area under the receiver operating characteristic (ROC) curve was calculated using the variables for *F. nucleatum* DNA and LINE-1 methylation. In this study, patients were followed up as outpatients every 1–3 months after discharge until death or December 2021. Cancer-specific survival was defined as the period from the date of surgery to the date of death by esophageal cancer. The Kaplan–Meier method was used to describe the distribution of esophageal cancer-specific survival time, and the log-rank test was performed. Until the time of censoring, censored subjects are considered "at risk", and thus continue to contribute towards the calculation of percent survival. survival curve was accompanied by a table giving the actual numbers of patients involved. Interactions were evaluated using the Wald test for confounding the respiratory morbidity variable with another variable of interest.

Results

We utilized 306 cases of patients who underwent resection of esophageal cancer at Kumamoto University Hospital and qualified the relative amounts of *F. nucleatum* DNA in the esophageal cancer tissues using qPCR. We divided the patients into an *F. nucleatum*-negative group ($n=241$, 78.8%) and an *F. nucleatum*-positive group ($n=65$, 21.2%), according to their *F. nucleatum* DNA status. There was no significant difference in the clinicopathological features of the patients in terms of age, sex, preoperative performance status, alcohol, smoking, and preoperative therapy (all $P>0.05$); however, the advanced stage was significantly

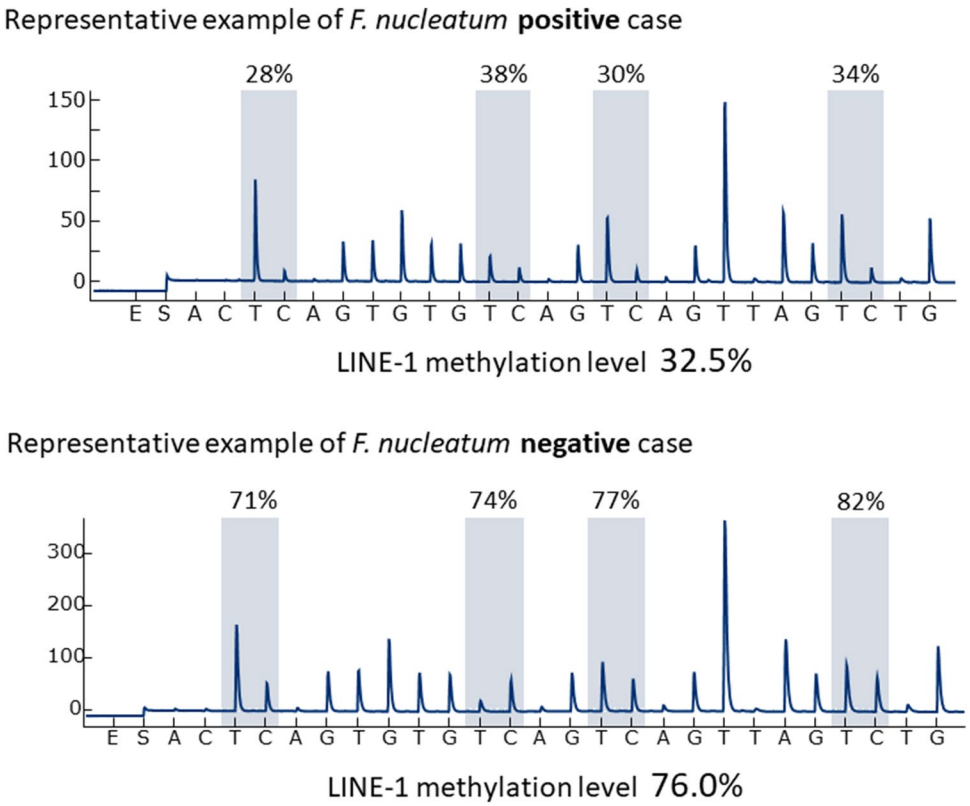


Fig. 1 Pyrosequencing assay used to measure the LINE-1 methylation level. Upper panel shows LINE-1 hypomethylated tumor (methylation level, 32.5). Lower panel shows LINE-1 hypermethylated tumor (methylation level, 76.0). The % (in blue) are the proportion of C at each CpG site after bisulfite conversion, and the methylation level of each CpG site was estimated by the proportion of C (%). The overall LINE-1 methylation level was calculated as the average of the proportions of C (%) at the 4 CpG sites

associated with *F. nucleatum* positivity ($P = 0.0023$) (Table 1). We have already reported in a previous paper that *F. nucleatum* affects the prognosis of esophageal cancer [13]. Similarly, in this cohort, we found that *F. nucleatum*-positive esophageal cancer cases had a significantly poorer prognosis than negative cases [log-rank $P = 0.0046$; univariate hazard ratio (HR) = 2.07, 95% confidence interval (CI) 1.22–3.41, $P = 0.0082$] (Supplemental figure).

We also measured the LINE-1 methylation level in the tumor lesions using pyrosequencing technology (Fig. 1). The distribution of the LINE-1 methylation level in the 306 cases was as follows: mean, 62.2; median, 62.0; SD, 12.7; range, 26.9–91.8; interquartile range, 53.3–71.4 (all 0–100 scale). LINE-1 methylation levels were distributed approximately normally. There was no significant correlation between LINE-1 methylation and any of the clinical features (age, sex, preoperative performance status, alcohol, smoking, or preoperative therapy). In this study, cases with methylation levels higher than median value 62.0 were classified as hypermethylated cases and those with lower levels as hypomethylated cases. In this cohort, LINE-1 hypomethylated cases had a predominantly poorer prognosis than hypermethylated cases (log-rank $P = 0.027$; univariate HR = 1.73, 95% CI 1.06–2.86, $P = 0.027$) (Supplemental figure).

Based on our hypothesis that the level of LINE-1 methylation in esophageal cancer tissues might be influenced by *F. nucleatum*, we next examined the relationship between *F. nucleatum* and LINE-1 methylation in esophageal cancer. We found that the positivity of *F. nucleatum* DNA in a tumor was significantly associated with LINE-1 hypomethylation ($P < 0.0001$ by paired *t* test) (Fig. 2A). Similar results were obtained when squamous cell carcinoma and adenocarcinoma were analyzed separately [$P < 0.001$ for squamous cell carcinoma (Fig. 2B); $P = 0.013$ for adenocarcinoma and others (Fig. 2C)]. In addition, the level of *F. nucleatum* DNA (as a continuous variable) was related to the LINE-1 methylation level ($P = 0.011$) (Fig. 2D). The ROC curve analysis showed that the area under the curve was 0.71, $P < 0.0001$ for detecting *F. nucleatum* positivity in a tumor (Fig. 3). These findings certainly support the clear relationship between *F. nucleatum* and LINE-1 hypomethylation in esophageal cancers.

We determined whether the influence of *F. nucleatum* on cancer-specific survival was modified by LINE-1 hypomethylation. We found that the effect was not significantly modified by LINE-1 hypomethylation (P for interaction = 0.34). In the LINE-1 hypermethylated cases, the *F. nucleatum*-positive cases experienced significantly shorter cancer-specific survival than the *F. nucleatum*-negative cases (log-rank $P = 0.0049$; univariate HR = 3.09, 95% CI 1.27–6.84, $P = 0.015$) (Fig. 4A). In the LINE-1 hypomethylated cases, the *F. nucleatum*-positive cases experienced shorter cancer-specific survival than the *F. nucleatum*-negative cases, though this was not statistically

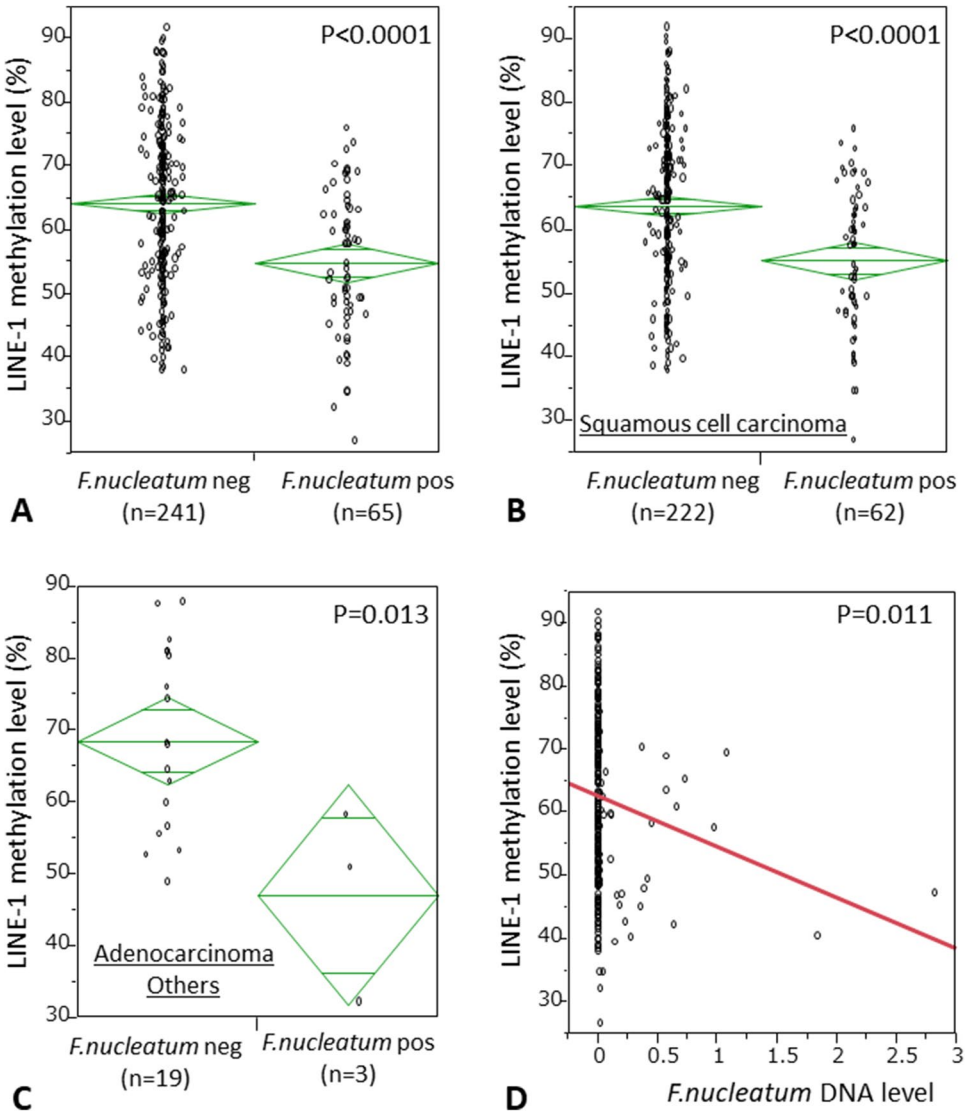


Fig. 2 **A** Assessment of LINE-1 methylation scores in the *Fusobacterium nucleatum*-negative and -positive groups in the tumor tissue of 306 patients with esophageal cancer. *P* values were determined using a *t* test. **B** Correlation between the tumor LINE-1 methylation level and the amount of *F. nucleatum* DNA in esophageal squamous cell carcinoma. **C** Correlation between the tumor LINE-1 methylation level and the amount of *F. nucleatum* DNA in esophageal adenocarcinoma and other cancers. **D** Correlation between the tumor LINE-1 methylation level and the amount of *F. nucleatum* DNA (as a continuous variable) in esophageal cancer

significant (log-rank $P=0.32$; univariate HR = 1.39, 95% CI 0.70–2.64, $P=0.33$] (Fig. 4B).

To test whether *F. nucleatum* specifically alters LINE-1 methylation in cancer cells, we evaluated the relationship between *F. nucleatum* and the methylation of *MGMT* and *MLH1* promoter region. We obtained valid results for *MGMT* methylation in 181 cases and for *MLH1* methylation in 182 cases using pyrosequencing technology and found that there was no significant relationship with *F. nucleatum* positivity ($P=0.12$ for *MGMT* methylation level and $P=0.66$ for *MLH1* methylation level) (Fig. 5). This indicates that *F. nucleatum* has a specific effect on LINE-1 methylation level (i.e., global DNA hypomethylation).

Discussion

We conducted this study to examine the relationship between *F. nucleatum* (a bacterial species in the gut microbiome) and LINE-1 hypomethylation (i.e., global DNA hypomethylation) among 306 patients with resected esophageal cancers. We could demonstrate that *F. nucleatum* DNA was significantly related to the LINE-1 hypomethylation of tumor lesions in esophageal cancer, but not to the methylation of tumor suppressor genes (i.e., *MGMT* and *MLH1*) promoter region. Increasing evidence suggests that both the gut microbiome and epigenetic changes play

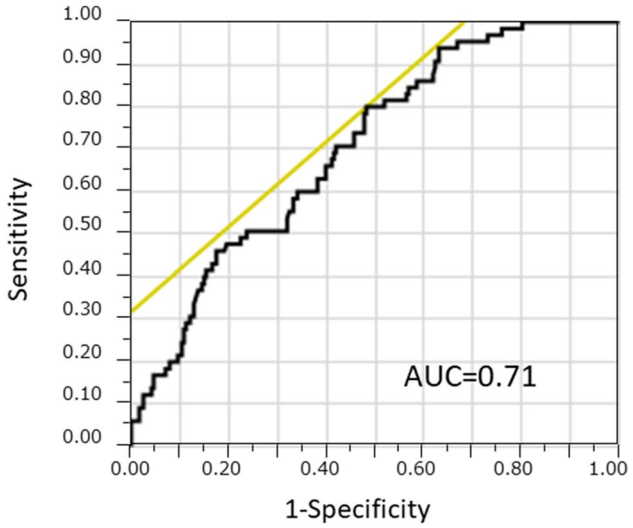
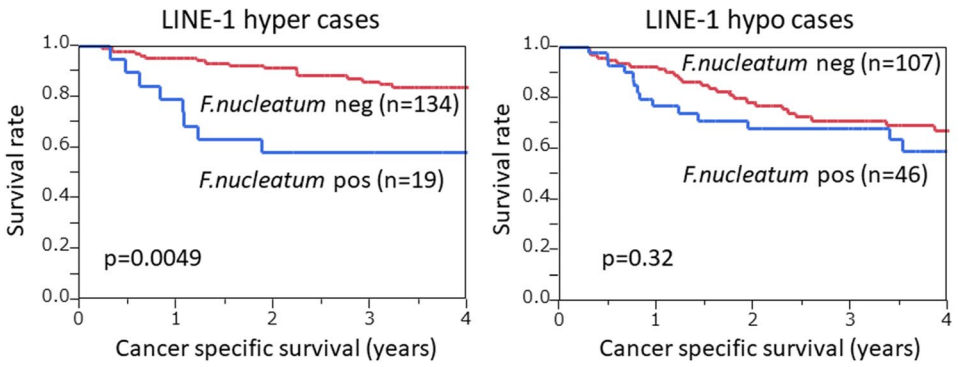


Fig. 3 ROC analysis was analyzed based on the amount of *Fusobacterium nucleatum* DNA and the LINE-1 methylation level in esophageal cancer

important roles in esophageal cancer development and could be therapeutic targets. Importantly, the gut microbiome can alter DNA methylation in host cells through a variety of mechanisms. To the best of our knowledge, this is the first study showing the relationship between *F. nucleatum* and LINE-1 hypomethylation in human cancers.

F. nucleatum is a gram-negative anaerobic bacterium found in the human oral and gastrointestinal tract [7]. *F. nucleatum* is an opportunistic pathogen, not only involved in inflammatory processes, such as periodontitis, inflammatory bowel disease, pancreatic abscess, premature birth, and liver abscess, but also involved in the progression of cancer [8]. The ability of *F. nucleatum* to adhere to epithelial cells might be one of the possible reasons that it promotes tumor development [10–12]. The abundance of *F. nucleatum* correlates with poor prognosis in patients with gastrointestinal cancer, further supporting its role in imparting aggressive tumor phenotype [8]. We previously reported that *F. nucleatum* is associated with shorter survival and an inferior chemotherapeutic response in esophageal cancer, suggesting its potential role as a prognostic or predictive biomarker [13, 22]. In addition, utilizing in vitro and in vivo models, we have demonstrated that *F. nucleatum* invaded esophageal cancer cells and induced the NF-κB pathway through the NOD1/RIPK2 pathway, leading to tumor progression [23]. NOD1 is a member of the NOD-like receptor family of proteins, which functions to detect peptidoglycan and stimulate host responses to limit bacterial infection. We are of course aware that *F. nucleatum* may affect esophageal cancer malignancy through various complex mechanisms other than this pathway. In particular, the recent finding that the gut microbiota influences DNA methylation in host cells led us to design this study.

In cancer cells, DNA methylation can be changed in two ways: global DNA hypomethylation and site-specific CpG island promoter hypermethylation. LINE-1 constitutes approximately 17% of the human genome, and its methylation level is well-correlated with the global DNA methylation status [15]. LINE-1 methylation is highly variable, and the strong relationships between LINE-1 hypomethylation and unfavorable prognosis have been shown in many types of human cancers, such as esophageal cancer [15–18]. We have previously demonstrated the prognostic impact of LINE-1 hypomethylation in esophageal cancer, supporting its potential role as a prognostic marker [18]. We also found that LINE-1-hypomethylated tumors showed highly frequent genomic gains at various loci containing candidate oncogenes, such as CDK6 [24]. These findings certainly support the importance of global DNA hypomethylation



Years	0	1	2	3	4
F. nucleatum (-)	134	106	92	76	49
F. nucleatum (+)	19	15	11	9	8

A

Years	0	1	2	3	4
F. nucleatum (-)	107	77	57	44	31
F. nucleatum (+)	46	28	22	19	9

B

Fig. 4 K–M curves according to the *Fusobacterium nucleatum* status in the LINE-1 hypermethylated cases (**A**) and LINE-1 hypomethylated cases (**B**)

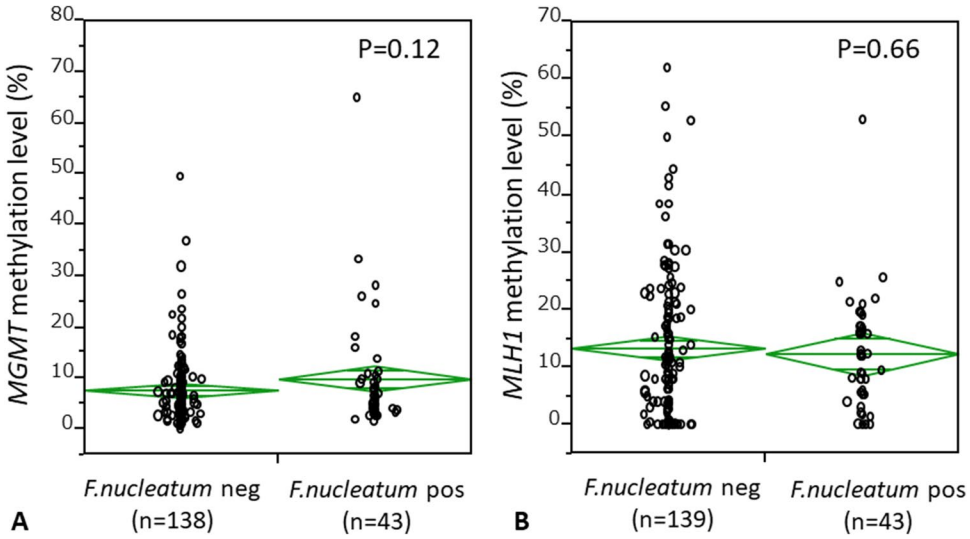


Fig. 5 **A** Relationship between *MGMT* promoter methylation level and the amount of *F. nucleatum* DNA. **B** Relationship between *MLH1* promoter methylation level and the amount of *F. nucleatum* DNA

(i.e., LINE-1 hypomethylation) in esophageal cancer development. Importantly, in this study, we demonstrated that *F. nucleatum* affects genome-wide methylation levels, but not promoter region methylation levels of tumor suppressor genes (*MGMT* and *MLH1*). Mismatch repair is one of the main DNA repair systems that relates to the homologous MutLS bacterial system (human MutS and MutL proteins) [25]. MLH1 (mutL homolog 1) is a human gene that plays a key role in the DNA duplication error reparation process, and likewise, it also plays a pivotal role in preserving genomic stability. Methylguanine–DNA methyltransferase (MGMT) is a specific DNA damage repair protein which plays a key role in maintaining normal cell physiology and genomic stability. Methylation of this promoter is a key predictor of whether alkylating agents can effectively control tumor cell progression [26]. It has been reported that promoter hypermethylation can silence these tumor suppressor genes, thereby contributing to the development of esophageal cancer. Of course, we understand that further validation is needed to determine whether *F. nucleatum* does not affect methylation of promoter regions of other cancer-related genes. In addition, further validation is needed to determine whether the relationship with LINE-1 methylation is *F. nucleatum*-specific or can occur in other oral bacteria.

Experimental studies have demonstrated that the gut microbiota plays an important role in the DNA methylation of host cells. Sobhani revealed that colorectal cancer-associated microbiota could induce the methylation of host genes, which contribute to epigenetic modification [27]. Kim et al. also found that the composition of the gut microbiome could play a role in persistent epigenetic modification of the liver [28]. Hattori et al. used an antibiotic to prohibit the tumorigenesis of colorectal cancer through aberrant DNA methylation induced by inflammation [29]. Although studies that focus on the link between *F. nucleatum* and epigenetic changes in human malignancies are rare, a population-based study by Koi et al. reported that *F. nucleatum* was associated with genomic hypermutation independent of the CpG island methylator phenotype (CIMP) and *BRAF* mutations [30]. Another study showed the relationship of *Fusobacterium* with wild-type *TP53*, *hMLH1* methylation, genomic hypermutation, *CHD7/8* mutation and the CIMP phenotype [31]. These studies strongly suggest the contribution of *Fusobacterium* to epigenetic alterations. This is the first study, to our knowledge, to reveal the relationship between the gut microbiome and epigenetic alterations in esophageal cancers. In this study, we demonstrated that the amount of intratumoral *F. nucleatum* DNA was related to the LINE-1 methylation of tumor lesions in esophageal cancer. This result may suggest that *F. nucleatum* may promote the development of esophageal cancer by regulating genome-wide DNA methylation. Nonetheless, we acknowledge that it is not clear how LINE-1 hypomethylation (i.e., genome-wide hypomethylation) is mediated in the process by which *F. nucleatum* affects the prognosis of esophageal cancer. We have demonstrated that *F. nucleatum* contributes to esophageal cancer malignancy via the NF-κB pathway, but we also believe that it influences cancer progression by other different mechanisms. We acknowledge that further validation is needed in this regard. In addition, the relationship between the localization of *F. nucleatum* in tumor tissue and the level of LINE-1 methylation should also be examined in the future.

These results highlight the epigenetic modification induced via gut microbiota modulation as a potential mechanism for exploring the pathogenesis of esophageal cancer. As the diagnostic and therapeutic applications of host cell epigenetic modification regulated by the gut microbiota have become increasingly popular, our study may provide information for pursuing targeted approaches for the etiology of esophageal cancer. In addition, considering that both gut

microbiota and DNA methylation are reversible and have attracted attention as targets for disease therapy, we believe that this study has clinical significance.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10388-023-01009-9.

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Data availability All presented data are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors have no conflict of interest.

Ethical approval This study was approved by the institutional review board of Kumamoto University (#1272). Written informed consent was obtained from all patients. Our study was performed as per the principles of the Declaration of Helsinki.

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