Fusobacterium nucleatum in gastroenterological cancer: Evaluation of measurement methods using quantitative polymerase chain reaction and a literature review

KENSUKE YAMAMURA[1], YOSHIFUMI BABA[1], KEISUKE MIYAKE[1], KENICHI NAKAMURA[1], HIRONOBU SHIGAKI[1], KOSUKE MIMA[1], JUNJI KURASHIGE[1], TAKATSUGU ISHIMOTO[1], MASAAKI IWATSUKI[1], YASUO SAKAMOTO[1], YOICHI YAMASHITA[1], NAOYA YOSHIDA[1], MASAYUKI WATANABE[2] and HIDEO BABA[1]

[1]Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8556; [2]Department of Gastroenterological Surgery, Cancer Institute Hospital, Japanese Foundation for Cancer Research, Tokyo 135-8550, Japan

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Abstract. The human microbiome *Fusobacterium nucleatum*, which primarily inhabits the oral cavity, causes periodontal disease and has also been implicated in the development of colorectal cancer. However, whether *F. nucleatum* is present in other gastroenterological cancer tissues remains to be elucidated. The present study evaluated whether quantitative polymerase chain reaction (qPCR) assays were able to detect *F. nucleatum* DNA and measure the quantity of *F. nucleatum* DNA in esophageal, gastric, pancreatic and liver cancer tissues. The accuracy of the qPCR assay was determined from a calibration curve using DNA extracted from cells from the oral cavity. Formalin-fixed paraffin-embedded (FFPE) tumor tissues from 20 patients with gastroenterological [esophageal (squamous cell carcinoma), gastric, colorectal, pancreatic and liver] cancer and 20 matched normal tissues were evaluated for *F. nucleatum* DNA content. The cycle threshold values in the qPCR assay for *F. nucleatum* and solute carrier organic anion transporter family member 2A1 (reference sample) decreased linearly with the quantity of input DNA (r^2>0.99). The *F. nucleatum* detection rate in esophageal, gastric and colorectal cancer tissues were 20% (4/20), 10% (2/20) and 45% (9/20), respectively. *F. nucleatum* was not detected in liver and pancreatic cancer tissues. The qPCR results from the frozen and FFPE tissues were consistent. Notably, *F. nucleatum* was detected at a higher level in superficial areas compared with the invasive areas. *F. nucleatum* in esophageal, gastric and colorectal cancer tissues was evaluated by qPCR using FFPE tissues. *F. nucleatum* may be involved in the development of esophageal, gastric and colorectal cancer.

Correspondence to: Professor Hideo Baba, Department of Gastroenterological Surgery, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan
E-mail: hdobaba@kumamoto-u.ac.jp

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Introduction

As a developing research area, the microbiome has been the focus of multiple studies in previous years. The non-spore-forming, anaerobic gram-negative bacterium *Fusobacterium nucleatum* is part of the normal flora of the human oral cavity and gut mucosa, but is an established opportunistic pathogen in periodontal diseases (1-4) and several inflammatory diseases, including inflammatory bowel disease (5-8), liver abscesses (9,10) and chorioamnionitis (11). Two previous studies have reported an overabundance of *F. nucleatum* in colorectal cancer tissues compared with adjacent normal tissues (12,13). Following this, a previous study demonstrated that *F. nucleatum* activates the E cadherin/β-catenin signaling pathway via FadA adhesion, promoting colorectal cancer growth (14). *Fusobacterium* subspecies (spp.), including *F. nucleatum,* are also present at increased levels in human colorectal, pancreatic and other types of cancer (12,13,15-20). To the best of our knowledge, there are only five previous studies reporting the presence of *Fusobacterium* spp. in colorectal and pancreatic cancer tissues and there are no published studies that associate *Fusobacterium* spp. with esophageal, gastric, hepatocellular and other gastroenterological cancer (Table I) (15,16,19,20,21).

Elevated levels of *F. nucleatum* DNA in colorectal cancer tissue are associated with certain molecules and cell functions, including microsatellite instability, the CpG island methylator phenotype and hMLH1 (15), and are also associated with a lower density of T cells (16). A number of previous studies have associated high levels of *F. nucleatum* DNA content with poor patient prognosis (17,18), however, other previous studies have reported that there is no association between the quantity of *F. nucleatum* DNA and patient survival rate (12,19). In one previous study, the DNA status of *Fusobacterium* spp. in pancreatic cancer tissue was independently associated with the poor prognosis of patients (20).

However, whether *F. nucleatum* is present in other types of gastroenterological cancer, including esophageal, gastric or liver cancer, has yet to be investigated.

In the present study, quantitative polymerase chain reaction (qPCR) method was evaluated to determine if it was able to detect the quantity of *F. nucleatum* DNA from an oral cavity. Subsequently, a qPCR assay was also used to analyze whether it similarly detects the existence of *Fusobacterium* in formalin-fixed paraffin-embedded (FFPE) tissues and frozen tissues. Finally, the quantity of *F. nucleatum* DNA in 20 paraffin-embedded digestive cancer specimens and 20 matched normal specimens was evaluated.

Materials and methods

Tissue samples. The test specimens were 20 FFPE tissue samples of esophageal (squamous cell carcinoma), gastric, colorectal, liver and pancreatic cancer, and 20 normal matched paraffin embedded specimens. All specimens were obtained by surgical resection at Kumamoto University Hospital (Kumamoto, Japan). The sampled patients were not administered preoperative treatment. A single pathologist, who was blind to the clinical and molecular data of the patients, evaluated hematoxylin-eosin-stained tissue sections of each cancer case and recorded the pathological features. Tumor staging was conducted as described in the Cancer Staging Manual (7th edition) published by the American Joint Committee on Cancer (22). Written informed consent was obtained from each patient and the present study was approved by the Institutional Review Board of Kumamoto University Hospital (Approval no. 1272).

DNA extraction and qPCR for F. nucleatum DNA content. Genomic DNA in the oral cavity was obtained using a cotton swab. The patients were not allowed to eat or drink 30 min prior to sample collection and the cotton swap was scraped against the inside of each cheek 5-6 times. The collected swab was air-dried for >2 h. The genomic DNA from the oral cavity was extracted using QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany). Genomic DNA from the FFPE tissues and from the frozen gastroenterological cancer tissues was extracted using the QIAamp DNA FFPE Tissue kit (Qiagen GmbH) and the QIAamp DNA Mini kit (Qiagen GmbH), respectively. The *nusG* gene of *F. nucleatum* and the reference human gene solute carrier organic anion transporter family member 2A1 (SLCO2A1) were amplified using custom-made TaqMan primer/probe sets (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) as previously described (18). The primer and probe sequences used for the custom TaqMan Gene Expression assay were as follows: *F. nucleatum* forward primer, 5'-TGG TGTCATTCTTCCAAAAATATCA-3'; *F. nucleatum* reverse primer, 5'-AGATCAAGAAGGACAAGTTGCTGAA-3'; *F. nucleatum* FAM probe, 5'-ACTTTAACTCTACCATGT TCA-3'; SLCO2A1 forward primer, 5'-ATCCCCAAAGCA CCTGGTTT-3'; SLCO2A1 reverse primer, 5'-AGAGGCCAA GATAGTCCTGGTAA-3'; SLCO2A1 VIC probe, 5'-CCATCC ATGTCCTCATCTC-3'. The PCR mix consisted of 1X TaqMan Environmental Master Mix 2.0 (Applied Biosystems; Thermo Fisher Scientific, Inc.), 0.5 pmol forward and reverse primer, 0.1 pmol probe, nuclease-free water (Invitrogen; Thermo Fisher Scientific, Inc.) and 12.5 ng genomic DNA in a total volume of 10 μl. Assays were performed in a 384-well optical PCR plate. The DNA was amplified and detected with the LightCycler 480 Instrument II (Roche Diagnostics, Basel, Switzerland) under the following reaction conditions: Initial denaturation at 95˚C for 10 min, 15 sec at 95˚C and 60 sec at 60˚C. The quantity of *F. nucleatum* DNA in each tissue was normalized relative to SLCO2A1 using the $2^{-\Delta\Delta Cq}$ method (where ΔCq is the mean Cq of *F. nucleatum* minus the mean Cq of SLCO2A1) (16,23). All RT-qPCR reactions were performed in triplicate.

Statistical analysis. All statistical analyses were performed by the JMP program, version 10 (SAS Institute, Inc., Cary, NC, USA). All P-values were 2-sided. The mean quantity of *F. nucleatum* DNA was compared with paired Student's *t*-tests for variables with two categories. P<0.05 was considered to indicate a statistically significant difference.

Results

Literature review. An online search of MEDLINE (PubMed) was performed for all articles published in the English language. The following Medical Subject Headings terms were used in combination: 'Fusobacterium esophageal cancer', 'Fusobacterium gastric cancer', 'Fusobacterium colorectal cancer', 'Fusobacterium hepatocellular carcinoma', and 'Fusobacterium pancreatic cancer'. The latest search was performed on December 2015. Among them, five studies which had detection rates of Fusobacterium spp. in cancer tissues were identified. In total, four previous studies have reported detectable levels of *F. nucleatum* in colorectal cancer tissues (15,16,19,21). The *F. nucleatum* detection rate was 13-82% in colorectal tumor tissue and 3.4-81% in adjacent normal tissue (Table I). A single previous study detected *F. nucleatum* in pancreatic cancer (the detection rate was 8.8% in tumor tissue and 28% in adjacent normal tissue) (20). However, the expression status of *Fusobacterium* DNA in esophageal, gastric and liver cancer remains to be elucidated.

Validation of qPCR for F. nucleatum. A cheek swab from a healthy researcher (Dr Kensuke Yamamura, Department of Gastroenterology, Kumamoto University, Kumamoto, Japan) was submitted for genomic DNA determination of the oral cavity. *F. nucleatum* and SLCO2A1 in the oral cavity were evaluated using qPCR in a 2-fold dilution series (5, 10, 12.5, 20 and 40 ng). The assays were quantified using the coefficient of determination (r^2) between 5 and 40 ng. In the qPCR assays of oral *F. nucleatum* and SLCO2A1, the cycle threshold (Cq) values linearly decreased with the quantity of input DNA (on a linear-log scale, r^2>0.99; Fig. 1). These results demonstrated that qPCR has the ability to quantify *F. nucleatum* DNA in the oral cavity.

qPCR of F. nucleatum in frozen tissue and FFPE. *F. nucleatum* DNA in FFPE and frozen tissues of 10 esophageal squamous cell carcinoma (ESCC) cases were investigated. In the 5 tissues that were positive for *F. nucleatum*, the organism was also detected in the matched FFPE tissues. Similarly, in the 5 *Fusobacterium*-negative ESCC cases, *F. nucleatum* was not detected in the matched FFPE tissue (Table II). Therefore, the

Table I. Detection rates of *Fusobacterium* spp. in gastroenterological cancer tissues from previous studies.

Authors	Type of cancer	No. of cases	Tissue fixation	Bacterial strain	*Fusobacterium* detection rate, %		(Refs.)
					Tumor tissue	Normal tissue	
Tahara *et al*, 2014	Colorectal cancer	149	Frozen tissue	*F. nucleatum*	52.3 (78/149)	30.3 (27/89)	(15)
Mima *et al*, 2015	Colorectal cancer	598	FFPE	*F. nucleatum*	13 (76/598)	3.4 (19/558)	(16)
Ito *et al*, 2015	Colorectal cancer	511	FFPE	*F. nucleatum*	56 (286/511)	-	(19)
Mitsuhashi *et al*, 2015	Pancreatic cancer	283	FFPE	*Fusobacterium species*	8.8 (25/283)	28 (7/25)	(20)
Viljoen *et al*, 2015	Colorectal cancer	71	FFPE	*F. nucleatum spp. polymorphum*	82 (58/71)	81 (48/59)	(21)

spp., subspecies; FFPE, formalin-fixed paraffin-embedded tissues; *F., Fusobacterium*.

Table II. Consistency of quantitative polymerase chain reaction detection of *Fusobacterium nucleatum* in tumor FFPEs and frozen tissues of esophageal cancer.

Variable	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10
FFPE	-	-	-	-	-	+	+	+	+	+
Frozen tissue	-	-	-	-	-	+	+	+	+	+
Concordance	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

FFPE, formalin-fixed paraffin-embedded tissues.

Table III. Quantitative polymerase chain reaction results of *Fusobacterium nucleatum* in gastroenterological cancer and adjacent normal tissues.

Type of cancer	Fusobacterium detection rate, %		
	Tumor tissue	Normal tissue	Tumor and normal tissues
Esophageal cancer	20 (4/20)	5 (1/20)	0
Gastric cancer	10 (2/20)	0	0
Colorectal cancer	45 (9/20)	40 (8/20)	25 (5/20)
Liver cancer	0	0	0
Pancreatic cancer	0	0	0

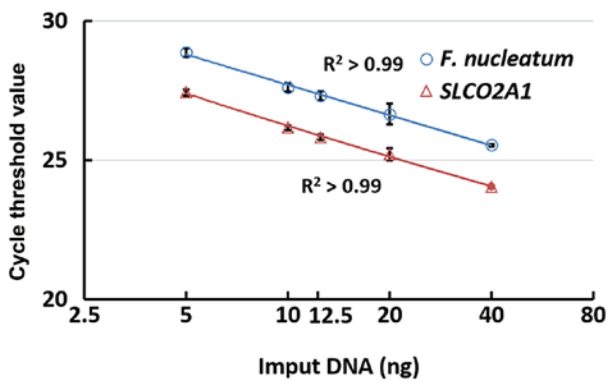

Figure 1. Analysis of linearity in quantitative polymerase chain reaction assay of *F. nucleatum* from the oral cavity. Levels of *F. nucleatum* and the human reference gene (SLCO2A1) from the oral cavity obtained by oral swab, demonstrated in a 2-fold dilution series (5, 10, 12.5, 20 and 40 ng). Data points and error bars denote the mean ± standard deviation of the cycle thresholds, respectively. Results were performed with triplicate runs in three separate experiments. *F., Fusobacterium*; R^2, coefficients of determination; SLCO2A1, solute carrier organic anion transporter family member 2A1.

qPCR results were consistent between the frozen tissues and FFPE tissues. These results suggested that *F. nucleatum* may be accurately assayed in FFPE tissues.

F. nucleatum in gastroenterological cancer tissue. 20 FFPE tumors and their adjacent non-tumorous tissues in each cancer were analyzed using qPCR assays. *F. nucleatum* was detected in 4 (20%) cases of esophageal cancer, 2 (10%) cases of gastric cancer and 9 (45%) cases of colorectal cancer (Fig. 2; Table III). In esophageal and colorectal cancer, *F. nucleatum* was also detected in adjacent non-tumor tissue, whereas *F. nucleatum* was not detected in the liver and pancreatic cancer tissues

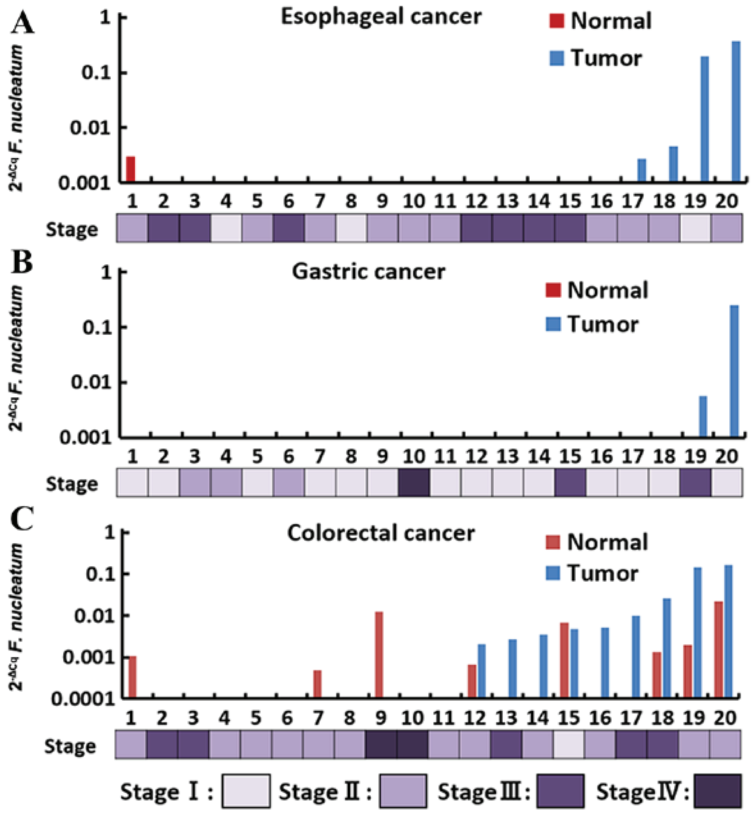

Figure 2. Relative quantity of *F. nucleatum* DNA in gastroenterological cancer. Quantitative polymerase chain reaction assays of *F. nucleatum* in the tumor and adjacent non-tumor tissues of 20 patients with (A) esophageal cancer, (B) gastric cancer and (C) colorectal cancer. The cancer stage of each tumor is also presented. *F.*, *Fusobacterium*.

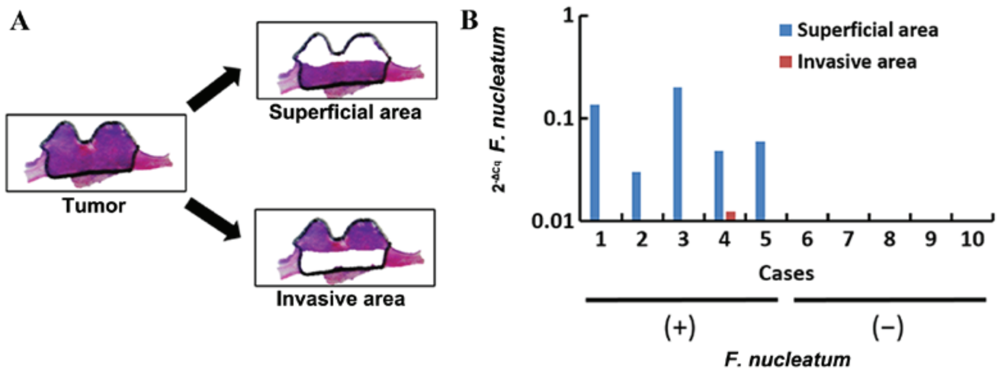

Figure 3. *F. nucleatum* DNA in superficial and invasive tissue areas. (A) Macrodissection of the superficial and invasive areas in tissues that were positive (cases 1-5) and negative (cases 6-10) for *F. nucleatum*. (B) *F. nucleatum* DNA was observed at high levels in superficial areas (range 30.1×10^{-3}-200.3×10^{-3}) and at low levels in invasive areas (0-12.4 $\times 10^{-3}$; P=0.02). *F.*, *Fusobacterium*.

and their adjacent non-tumor tissues. Among all cancer cases that were positive for *F. nucleatum*, the level of *F. nucleatum* DNA content in esophageal and colorectal cancer ranged from 2.68×10^{-3} to 365.2×10^{-3} (median, 101.3×10^{-3}) and from 2.10×10^{-3} to 166.7×10^{-3} (median, 5.08×10^{-3}), respectively.

Heterogeneity of F. nucleatum in esophageal cancer tissue. To evaluate the heterogeneity of the *F. nucleatum* DNA within tumor tissues, the *F. nucleatum* DNA in the superficial and invasive areas of the 5 esophageal cancer tissues that were positive for *F. nucleatum* were evaluated (Fig. 3A). High levels of *Fusobacterium nucleatum* DNA was observed in superficial areas, but low levels were observed in invasive areas. In the superficial areas, the quantity of *F. nucleatum* DNA ranged from 30.1×10^{-3} to 200.3×10^{-3}, whereas in invasive areas it was 12.4×10^{-3} at its highest (P=0.02; Fig. 3B). Therefore, the *F. nucleatum* DNA may distribute heterogeneously within a single tumor.

Discussion

F. nucleatum has received increased recognition as an opportunistic pathogen in periodontal diseases, but also in human cancer. As the microbiome has a number of important effects on the functions of the human body, the clinical significance of the discovery of *F. nucleatum* cannot be overemphasized. To the best of our knowledge, the present study has reported the first detection of *F. nucleatum* DNA in esophageal, gastric and liver cancer tissues. The present study has demonstrated that the qPCR assay may reliably detect *F. nucleatum* DNA from oral swabs, as *F. nucleatum* is among the most prevalent species in the oral cavity (1,2,24). The association between cycle threshold and input DNA in the qPCR assay of *F. nucleatum* was linear (r^2>0.99). Furthermore, the FFPE and frozen tissues prepared from the same esophageal tumor yielded a similar level of detection accuracy. Typically, the fixation process chemically alters the nucleic acids in a sample by inducing covalent DNA cross-linking and fragmentation. These alterations may reduce the efficacy of analysis using PCR and DNA sequencing methods (25,26). In the present study, the results of the FFPE and frozen tissues were concordant, which suggested that in the two types of tissue preparations, qPCR accurately detected *F. nucleatum* DNA in gastroenterological cancer tissues.

F. nucleatum DNA was successfully detected in gastrointestinal tract cancer tissues (esophageal, gastric and colorectal cancer), but *F. nucleatum* was not detected in pancreatic and liver cancer tissues. In previous studies, the detection rates of *F. nucleatum* were 13-82% in colorectal cancer (15,16,19,21) and 8.8% in pancreatic cancer (20). These previous studies are concordant with the data from the present study that uses colorectal cancer tissues, but these results contradict the pancreatic cancer results in the current study. Although *F. nucleatum* is part of the normal flora of the oropharyngeal and gastrointestinal tracts, *F. nucleatum* also expresses FadA, a bacterial cell surface adhesion protein that activates the WNT signaling pathway in colorectal cancer cells, and consequently promotes tumor growth (14). Therefore, it may reasonably be expected that the detection rate of *F. nucleatum* is higher in gastroenterological cancer compared with liver and pancreatic cancer. However, the presence of *F. nucleatum* in esophageal and gastric cancer tissues remains to be investigated.

In addition, the *F. nucleatum* expression levels in superficial and invasive areas of esophageal cancer tissues were compared, and an increased level was observed in superficial areas. This result suggested that *F. nucleatum* may not be able to infiltrate into the invasive area and may only contribute to the tumor growth through the side of the gastrointestinal tract. As the differential distribution of *F. nucleatum* has not been previously reported, the low level of *F. nucleatum* in invasive areas remains to be fully elucidated. The involvement of *F. nucleatum* in tumor growth requires further investigation.

In conclusion, *F. nucleatum* DNA was detected in esophageal, gastric and colorectal cancer, but not in pancreatic and liver cancer. This suggested that *F. nucleatum* may be associated with the progression of gastroenterological tract cancer, but not the progression of pancreatic and liver cancer.

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