Fusobacterium is enriched on the surface and surrounding duodenal mucosa of ampullary carcinoma

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Abstract

Background and Aims:

In colorectal cancer, adenomas develop into carcinomas through the adenoma–carcinoma sequence, which may be triggered by intestinal adherent microbial communities, including *Fusobacterium*. In this study, we aimed to explore the microbiome in ampullary tumors and clarify its relevance.

Methods:

Seventeen patients who underwent endoscopic retrograde cholangiopancreatography for ampullary tumors at Nagoya University Hospital from August 2020 to August 2021 were enrolled. The patients were divided into an adenoma group (n = 11) and a carcinoma group (n = 6). Ampullary samples were collected from the tumor surface (tumor samples, TSs) or surrounding normal duodenal mucosa (normal samples, NSs) via brush rubbing and then analyzed.

Results:

The Shannon $\alpha$-diversity index was considerably higher in the carcinoma group than in the adenoma group. *Fusobacterium*, *Leptotrichia*, *Methylorubrum*, and *Micrococcus* were enriched in the TSs of the carcinoma group. The relative abundance of *Fusobacterium* markedly increased with tumor progression. *Fusobacterium*, *Porphyromonas*, *Granulicatella*, and *Solobacterium* were enriched in the NSs of the carcinoma group, whereas *Bergeyella* was enriched in those of the adenoma group.

Conclusions:

Patients with ampullary carcinoma have a greater abundance of *Fusobacterium* in the tumor and surrounding normal duodenal mucosa than patients with adenoma.

INTRODUCTION

Ampullary tumors are rare, accounting for approximately 0.5% of gastrointestinal tumors [1]; however, their detection rate has increased with the extensive use of endoscopy [2]. Ampullary adenomas are precancerous lesions that require treatment [3]. The standard treatments include endoscopic papillectomy (EP) for adenomas [4] and pancreatoduodenectomy (PD) for carcinomas; however, adenomas are difficult to distinguish from carcinomas. The concordance rate between preoperative biopsy and postoperative pathology ranges from 62–85%, and approximately 30% of carcinomas cannot be diagnosed using biopsy [5]. Understanding the mechanisms underlying tumor progression is crucial for the resolution of the clinical questions mentioned above and for the discovery of new diagnostic [6, 7] and therapeutic methods.

In recent years, the relationship between gastrointestinal cancer and the microbiome has received considerable attention. In pancreatic cancer, specific intratumor bacteria were reported to modulating
drug metabolism properties [8]. In colorectal cancer (CRC), adenoma develops into carcinoma through the adenoma–carcinoma sequence, and compositional changes in gut adherent microbial communities may cause microbial dysbiosis, which may contribute to the development and progression of tumors [9]. Analyses of feces and digestive tissues using 16S rRNA sequencing revealed that several bacteria, including *Fusobacterium*, are responsible for dysbiosis [10]. *Fusobacterium* promotes colon oncogenesis through attachment, phosphorylation, and invasion [11]. In fact, *Fusobacterium* is known to be persistent CRC and liver metastases [12]. Ampullary adenomas also undergo the adenoma–carcinoma sequence [13], and the ampulla of Vater is constantly exposed to various digestive fluids, such as gastric, bile, and pancreatic juice. Hence, the microbiome is possibly associated with the progression of ampullary tumors, but substantial evidence supporting this theory is currently lacking. We aimed to clarify whether the gut microbiome is involved in the progression of ampullary tumors.

**PATIENTS AND METHODS**

**Study design and participant selection**

Seventeen patients who had undergone endoscopic retrograde cholangiopancreatography (ERCP) for ampullary tumors from August 2020 to August 2021 were enrolled. Informed consent was obtained from all the enrolled patients. The participants were subjected to blood tests, endoscopic observation of the tumor, and biopsies of one or two samples. ERCP and intraductal ultrasonography [14, 15] were used to evaluate intraductal extension. This study was approved by the Ethics Committee of Nagoya University Hospital (No. 2015–0420, August 30, 2016), and written consent was obtained from all participants prior to enrollment in accordance with the Declaration of Helsinki. This study was registered at the University Hospital Medical Information Network Clinical Trials Registry (UMIN ID: 000020269).

EP is suggested for the treatment of adenomas or carcinomas in adenomas without extension beyond the sphincter of Oddi, without signs of advanced cancer (strong redness or depression), without metastasis on CT scans, and with an adenoma component identified during the biopsy [16, 17]. PD is recommended for patients with gross findings that reflect signs of advanced cancer or when moderately or poorly differentiated adenocarcinoma is detected. Biopsies and specimens were evaluated by two or more pathologists, and eligible patients were divided into adenoma (n = 11) and carcinoma (n = 6) groups based on their postoperative pathology results. Patients were excluded if they had used antibiotics or immunosuppressive drugs within a month prior to the examination; a history of gastric or colorectal surgery; active infection; or uncontrolled cardiac, renal, or hepatic disease.

**Sample collection and 16S rRNA gene sequencing**

Samples were collected by rubbing over the tumor (tumor samples; TSs) and surrounding normal duodenal mucosa (normal samples; NSs) with an endoscopic cytology brush (COOK Medical, Tokyo, Japan) during ERCP. After rubbing, the tip of the brush was retracted to prevent it from sticking to any other part of the body, and samples were collected by cutting the brush connection with pliers after the tip
was removed from the body. After collection, all samples (n = 34) were quickly stored at -80 °C until analysis.

Microbiological analysis was performed following previously reported methods [18]. In brief, DNA was isolated using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany), and the V3–4 regions of bacterial 16S rRNA were amplified at 95°C using the following universal PCR primers (forward primer: 5' - TCGTCGGCAGCGGTGTATAAGAGACAGCCTACGACGACTACGGGNGGCWGCAG-3', reverse primer: 5' - GTCTCGTGGGCTCGAGATGTGAAGAGTCAGTCTACGGGAGATGTGTAAGACAGCCAGACAGGACTACHVGGGTATCTAAATCC-3'). The PCR products were pooled to construct a sequencing library. Then, they were sequenced using an Illumina MiSeq sequencer to generate paired-end reads using MiSeq Reagent Kit v3 for 2 x 300 reads and 600 cycles (Illumina, San Diego, CA, USA). The sequencing data were analyzed using the Quantitative Insights into Microbial Ecology 2 (QIIME2 version 2019.11 https://docs.qiime2.org/2020.11/) pipeline [19]. Feature table construction and sequence quality control were performed using Divisive Amplicon Denoising Algorithm 2 [20]. The SILVA database version 132 (Latin Silva, Forest, http://www.arb-silva.de) [21] was used to analyze the 16S rRNA sequencing results.

Statistical analysis

Categorical and continuous variables between the two groups were compared using Fisher’s exact test and the Mann–Whitney U test, respectively. Correlation coefficients were determined using Spearman’s rank correlation coefficient. The trend of continuous variables was analyzed using the Jonckheere–Terpstra test. Statistical analyses were performed using EZR version 1.53 [22]. Comparisons of microbiomes and their diversity were statistically analyzed and visualized using the online microbiome data analysis platform MicrobiomeAnalyst (https://www.microbiomeanalyst.ca/MicrobiomeAnalyst) [23]. The data used in MicrobiomeAnalyst were diluted to rarefy the minimum library size. The α-diversity was set at the genus level and calculated using the Chao1, Observed species, and Shannon indexes. The β-diversity was calculated using the ANOSIM (Bray-Curtis index), a nonparametric test, and the genus level was selected as the level of analysis. Comparisons were made using the Mann–Whitney U test. Differential taxonomy between the two groups was analyzed using linear discriminant analysis of effect size (LefSe) [24] provided by Galaxy (https://huttenhower.sph.harvard.edu/galaxy/) with a linear discriminant analysis score > 2 at the genus level. Statistical significance was set at p < 0.05.

RESULTS

Patient background

Patient background details are shown in Table 1. All patients had sporadic tumors and did not include familial adenomatous polyposis cases. None of the patients had intraductal extension; bile or pancreatic duct stents previously inserted; or symptoms such as jaundice, cholangitis, and pancreatitis. One case each of vasovagal reflex and severe pancreatitis was observed as an ERCP-related adverse event, and no complications due to brush rubbing were reported. Fourteen patients without obvious advanced cancer—which included eleven with adenoma, one with Tis carcinoma, and two with pT1a carcinoma—were
treated with EP. Three patients with gross depression, which included two patients with pT1b and one with T2, were treated with PD. The patients in the two groups showed no significant differences in age, sex, body mass index, tumor size, diabetes, use of proton pump inhibitors or statins, presence of atrophic gastritis, or blood test results. In addition, none of the patients were taking probiotics.
Table 1
Patient background

<table>
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<tr>
<th></th>
<th>Adenoma (n = 11)</th>
<th>Carcinoma (n = 6)</th>
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<td>Age (years, median, range)</td>
<td>69 [51–80]</td>
<td>67 [48–79]</td>
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<td>Sex, n</td>
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</tr>
<tr>
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<td>3</td>
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<td>BMI (median, range)</td>
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<td>19 [10–40]</td>
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<td>Statin, n</td>
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<td>Blood test findings (median, range)</td>
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<td>WBC (/µL)</td>
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<td>5350 [5100–7900]</td>
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<td>Hb (g/dL)</td>
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<td>14.3 [10.3–16.4]</td>
<td>&gt; 0.999</td>
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<td>Alb (g/dL)</td>
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<td>17.5 [12–32]</td>
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<tr>
<td>ALT (U/L)</td>
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Comparison of α and β diversity between adenoma and carcinoma groups

Results of 16S rRNA sequencing analysis revealed 3351 features in all samples. The median number of detected features was 34,172 (range, 8838–84,096) in the adenoma group and 25,297.5 (range, 4967–97237) in the carcinoma group, with no significant difference between the two groups (p = 0.444; Fig. 1a). The Shannon α-diversity index was significantly higher in the carcinoma group than in the adenoma group (p = 0.021), whereas the Chao1 (p = 0.481) and Observed species (p = 0.415) α-diversity indexes showed no significant differences (Fig. 1b). The analysis of β-diversity using the Bray-Curtis index showed no significant differences between the adenoma and carcinoma groups (R = 0.074227, p < 0.136; Fig. 1c).

Comparison of TSs between adenoma and carcinoma groups
The relative abundance of bacteria in the TSs was analyzed at the phylum (Fig. 2a) and genus (Fig. 2b) levels. In the adenoma group, *Firmicutes* (37.8%) was the most dominant phylum, followed by *Bacteroidota* (33.9%), and *Proteobacteria* (11.9%). In the carcinoma group, *Bacteroidota* (38.3%) was the most dominant, followed by *Firmicutes* (32.9%), and *Fusobacteriota* (15.4%). Furthermore, the relative abundance of *Fusobacteriota* was significantly greater in the carcinoma group than in the adenoma group (15.4% vs. 5.3%, p = 0.009). Figures 2b and 2d show the 44 most abundant genera of the 180 detected. *Prebotella* had the highest relative abundance in both groups (adenoma, 24.6%; carcinoma, 21.1%).

A comparison of the microbiome using LEfSe is shown in Fig. 3a. At the genus level, *Fusobacterium*, *Leptotrichia*, *Methylorubrum*, and *Micrococcus* had greater relative abundances in the carcinoma group than in the adenoma group. Bacteria with greater relative abundances in the adenoma group than in the carcinoma group were not identified. A trend test was performed to determine the relative abundance of *Fusobacterium* at each stage (Fig. 3b). The abundance of *Fusobacterium* increased significantly with tumor progression (p = 0.037).

### Comparison of NSs between adenoma and carcinoma groups

In the NSs of both groups, *Firmicutes* was the most dominant phylum (Fig. 2c), followed by *Bacteroidota*. However, the third most abundant phylum in the adenoma group was *Proteobacteria* (adenoma, 15.0%; carcinoma, 2.6%; p = 0.044), whereas that in the carcinoma group was *Fusobacteriota* (carcinoma, 12.2%; adenoma, 4.4%; p = 0.005). Furthermore, the most abundant genus in the adenoma group was *Streptococcus*, whereas that in the carcinoma group was *Prevotella* (Fig. 2d).

Figure 4 shows the bacteria with significantly different relative abundances between the two groups. The relative abundances of the genera *Fusobacterium*, *Porphyromonas*, *Granulicatella*, and *Solobacterium* were considerably greater in the carcinoma group than in the adenoma group. By contrast, the relative abundance of *Bergeyella* was greater in the adenoma group than in the carcinoma group.

### Correlation between Fusobacterium and other bacteria in ampullary tumors

In all 34 samples collected, the correlation between *Fusobacterium* and *Leptotrichia*, *Porphyromonas*, and *Granulicatella*, which were enriched in the carcinoma group as determined by LEfSe, is shown in a double logarithmic graph using scatter plots (Fig. 5). No significant difference in relative abundance was observed between *Fusobacterium* and *Leptotrichia* (Fig. 5a) or *Porphyromonas* (Fig. 5b), whereas the relative abundances of *Fusobacterium* and *Granulicatella* (Fig. 5c) showed a significantly positive correlation (p = 0.021).

**DISCUSSION**
Microbiome characterization showed that some bacteria, including *Fusobacterium*, had a greater relative abundance in the carcinoma group than in the adenoma group. The Chao1 and Observed species α-diversity indexes showed no significant difference between the adenoma and carcinoma groups. However, the Shannon α-diversity index, which evaluates community evenness, was considerably higher in the carcinoma group than in the adenoma group. The Chao1 and Observed species indexes focus on rare bacteria, whereas the Shannon index is strongly influenced by major bacteria, which often have great relative abundances. The differences in diversity scores were due to the higher diversity associated with major bacteria compared with rare bacteria. Major bacteria include autochthonous bacteria of the intestinal tract, such as *Bifidobacterium*, and oral bacteria, such as *Fusobacterium*. In this study, the TSs and NSs from the carcinoma group showed greater abundance of oral bacteria than those from the adenoma group, whereas similarities between the oral bacteria and the duodenal mucosal microbiota have been previously reported [25]. Meta-analyses revealed that the gut microbiome of patients with CRC is enriched with oral bacteria, such as *Fusobacterium*, *Porphyromonas*, and *Granulicatella*, and that the α-diversity in patients with CRC is higher than that in healthy controls [26]. Although oral samples were not collected in this study, bacterial species known to be endemic to the oral cavity were notably detected more frequently in the TSs of the carcinoma group. It is possible that the same increase in diversity may have occurred in ampullary tumors owing to the influx of oral commensal bacteria.

The mechanism underlying ampullary tumor progression has not yet been clarified, but the adenoma–carcinoma sequence has been reported to be one of the most promising factors. Kaiser *et al.* [13] reported that adenomatous lesions can be found in 65% of surgically resected adenocarcinomas and that the percentage of detected adenomatous lesions decreases considerably with progression to an advanced stage. Molecular mechanisms include mutations in the tumor suppressor gene p53, which is expressed more frequently in carcinomas with adenomatous areas than in adenomas [27]. In addition, K-ras mutations are found at higher rates in ampullary adenomas and adenocarcinomas [28]. Hayata *et al.* [29] reported that Axin2-expressing cells in the peribiliary glands surrounding the ampulla of Vater are cholangioepithelial stem/progenitor cells that can differentiate into ampullary carcinomas. Furthermore, Rsspondin3 secreted by the surrounding smooth muscle cells maintains the activation of Wnt/β-catenin signaling and contributes to tumor progression [27]. Genetic mutations and β-catenin signaling are important in the progression of ampullary tumors. In CRC, *Fusobacterium* reportedly plays a role in the adenoma–carcinoma sequence. It is more abundant in carcinomas than in adenomas [30] and expresses FadA adhesin, which contributes to tumor progression by activating β-catenin signaling through E-cadherin in epithelial cells and promoting the expression of Wnt genes and oncogenes [31]. Additionally, *Fusobacterium* activates Wnt/β-catenin signaling by upregulating CdK5, which is a member of the cyclin-dependent kinase family involved in cell cycle and transcription initiation and is associated with liver and colorectal cancers [32]. *Fusobacterium* is thought to be involved in carcinogenesis via β-catenin signaling activation. In the present study, the relative abundance of *Fusobacterium* in the TSs extensively increased with disease progression. This finding suggests that *Fusobacterium* is involved in the progression of ampullary carcinoma and colorectal cancer. *Leptotrichia* species are found in the blood of compromised patients and are thought to be pathogenic [33]. They are enriched in the mouths of patients with
pancreatic cancer [34] and are expected to serve as a biomarker for cancer detection [35]. In patients with CRC, Leptotrichia is co-enriched with Fusobacterium [36] and is a passenger bacterium [37]. In the driver–passenger model, a specific gut microbiome causes epithelial DNA damage and provides a basis for carcinogenesis. As the tumor progresses, a microenvironment favorable to the growth of opportunistic bacteria is created, thus defeating the driver bacteria and replacing them with passenger bacteria [38]. In the present study, the abundance of Leptotrichia increased with that of Fusobacterium, suggesting that Leptotrichia co-existed with Fusobacterium as a passenger bacterium.

The relative abundance of Fusobacterium was greater in the NSs of the carcinoma group than in those of the adenoma group. Previous studies have shown that the abundance of Fusobacterium in the colon increases in tumor and non-tumor areas with disease progression [39] and can combine with other oral bacteria to create biofilms [40]. Biofilms are formed through the outer membrane protein RadD of Fusobacterium [41], preventing other bacteria from attaching to and invading epithelial cells. Patients with biofilm-positive tumors have biofilms on non-tumor mucosa that are similar to those on the tumor [42]. Biofilms can also spread throughout the intestinal tract. Analyses of surgical specimens from patients with CRC showed that biofilms similar to those on the tumor area are as widespread throughout the resected specimens [43]. Fusobacterium with sufficient abundance on the tumor surface could spread to the surrounding healthy mucosa through biofilms. Porphyromonas and Granulicatella are abundant in saliva samples obtained from patients with pancreatic cancer. They are considered risk factors for pancreatic cancer [44] and are able to form biofilms with Fusobacterium [45]. In the present study, the relative abundance of Fusobacterium was greater in the NSs, the abundance of Fusobacterium and Porphyromonas tended to correlate, and Fusobacterium and Granulicatella were significantly correlated (p = 0.036).

To the best of our knowledge, this study is the first to evaluate the microbiome on the surface of ampullary tumors. However, this study has several limitations. Specifically, the study had a single-center design and a small sample size. Moreover, it lacked a normal control group. ERCP was not performed on healthy patients without any disease, and the possibility of pancreatitis was considered to be caused by rubbing of the papilla. Obtaining healthy samples is ethically difficult; hence, we compared the microbiota of adenomas and carcinomas. TSs and NSs must be examined separately to correlate Fusobacterium with Leptotrichia, Porphyromonas, and Granulicatella. However, considering that Leptotrichia, Porphyromonas, and Granulicatella can form biofilms with Fusobacterium, we analyzed all samples.

In conclusion, ampullary carcinomas are co-enriched with Fusobacterium and other bacteria that can form biofilms on the tumor and surrounding normal duodenal mucosa. Ampullary carcinoma could be associated with Fusobacterium and other bacteria during tumor progression, as evident in patients with CRC. The results of this study can serve as a reference for developing new diagnostic and therapeutic methods for ampullary carcinoma.

Declarations
Conflict of interests

There are no conflicts of interest to declare.

Funding information

None.

References


Figures
Figure 1

Comparison of the number of features (a), α-diversity (b) and β-diversity (c) between the adenoma and carcinoma groups
Figure 2

Proportion of the microbiome on the tumor surface at phylum (a) and genus (b) levels in the adenoma and carcinoma groups. Microbiome at the phylum (c) and genus (d) levels on the surrounding normal duodenal mucosa.
Figure 3

Comparison of relative abundance of bacteria between samples collected from the tumor surface in the adenoma and carcinoma groups via linear discriminant analysis (LDA) of effect size. (a) Bacteria shown with red bars indicating LDA score have significantly greater relative abundance in the carcinoma group than in the adenoma group. Significant differences are observed in the phylum *Fusobacteria*. (b) Relative
abundance of *Fusobacterium* at each invasion depth. Relative abundance increases with depth. Vertical bars indicate the range of *Fusobacterium* abundance.

**Figure 4**

Comparison of relative abundance of bacteria between samples collected from the normal duodenal mucosa in the adenoma and carcinoma groups via linear discriminant analysis of effect size. Bacteria
with red bars have significantly greater relative abundance in the carcinoma group than in the adenoma group. Bacteria with green bars have significantly greater relative abundance in the adenoma group than in the carcinoma group.

Figure 5

Scatter plots and correlations between the abundance of *Fusobacterium* and *Leptotrichia* (a), *Porphyromonas* (b), and *Granulicatella* (c). White circles indicate tumor samples, whereas black circles indicate normal samples.