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Reduction of butyrate-producing bacteria in the gut microbiome of Japanese patients with pancreatic cancer

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ABSTRACT

Background: The incidence of pancreatic cancer is on the rise, and its prognosis remains poor. Recent reports have established a link between the gut and oral microbiome and pancreatic cancer. However, the intricacies of this association within the Japanese population remain unclear. In this study, we investigated the gut and oral microbiomes of Japanese patients with pancreatic cancer, comparing them with those of healthy individuals.

Methods: We recruited 30 patients with untreated pancreatic cancer and 18 healthy controls at Kyoto University Hospital (2018–2022). We performed a comprehensive 16S rRNA gene sequencing to analyze their gut and oral microbiomes.

Results: Analysis revealed that the diversity of the gut and oral microbiomes of patients with pancreatic cancer was reduced compared to that of the healthy controls. Specifically, we observed an increase in the genus *Streptococcus* in both the gut and oral microbiomes and a significant decrease in several butyrate-producing bacteria in fecal samples. Moreover, bacteria such as *Streptococcus mitis* and *Holdemanella biformis* were present in pancreatic cancer tissues, suggesting that they might influence the carcinogenesis and progression of pancreatic cancer.

Conclusions: The gut and oral microbiome differed between patients with pancreatic cancer and healthy controls, with a notable decrease in butyrate-producing bacteria in the gut microbiome of the patients. This suggests that there may be a distinct microbial signature associated with pancreatic cancer in the Japanese population. Further studies are required to elucidate the microbiome's causal role in this cancer and help develop prognostic markers or targeted therapies.

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1. Introduction

Pancreatic cancer represents a significant global health challenge. Its incidence is on the rise, and the prognosis remains poor, with a 5-year survival rate of approximately 10 %, despite advancements in treatment options [1,2]. This highlights the need for a deeper understanding of the underlying biological mechanisms contributing to the initiation and progression of this disease.

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Microbiome is a collection of microorganisms including bacteria which live in each human organ. Dysbiosis of gut microbiome has been reported to be associated with diabetes, obesity, and cardiovascular disease [3–5]. Recent studies have suggested a potential correlation between the microbiome and the development of various types of cancer [6–12], including pancreatic cancer [13–16]. The interplay between the gut and oral microbiomes and pancreatic cancer is an emerging field of research. Our study is driven by the need to explore this further, particularly, in the Japanese population, and could be crucial for developing new prognostic or therapeutic approaches. Bacteria are more abundant in patients with pancreatic cancer than in healthy individuals [10]. Germ-free conditions have been shown to suppress the formation of pancreatic precancerous lesions (PanIN) in mouse models, while fecal transplantation from mice with pancreatic cancer into germ-free mice increased PanIN formation [10]. The pancreas is anatomically connected to the duodenum via the duodenal papilla through the pancreatic duct, and gut bacteria may migrate into the pancreas. In surgical cases of pancreatic cancer, patients with long-term survival exhibited significantly more diverse bacterial microbiome within the tumor microenvironment than those with shorter-term survival [14]. Additionally recent studies have reported the presence of detectable amounts of bacteria and fungi in human pancreatic cancer tissue [15,17].

A recent multinational study conducted in Japan has identified a microbial signature among patients with pancreatic cancer [18]. However, given that the microbiome differs among different races [19] and that Japanese have different gut and oral microbiome from other races [20], the relationship between the gut and oral microbiomes and pancreatic cancer in the Japanese population remains unclear. Therefore, this study aimed to elucidate the association between the gut and oral microbiomes and pancreatic cancer in Japanese patients.

2. Materials and methods

2.1. Study population and sample collection

The pancreatic cancer group consisted of 30 patients who were admitted for evaluation and treatment of untreated pancreatic cancer to the Department of Gastroenterology and Hepatology at Kyoto University Hospital, between 2018 and 2022. The healthy control group (controls) comprised 18 outpatients who participated in opportunistic screening at the Preemptive Medicine and Lifestyle-Related Disease Research Center (HIMEDIC), also at Kyoto University Hospital, during the same timeframe. Written informed consent was obtained from all study participants. This study adheres to the ethical standards outlined in the Declaration of Helsinki. The study protocol (Protocol no. R1844-2) was approved by the Ethics Committee of Kyoto University. For use in fluorescent in situ hybridization (FISH), 14 resected pancreatic cancer specimens were utilized. These samples were exclusively for this experiment, and the Ethics Committee approved their use with informed consent in the form of opt-out on the website.

2.2. Microbiome analysis

A comprehensive analysis of the microbiome was conducted through 16S rRNA gene sequencing. DNA was extracted from fecal samples using the bead-phenol method [21], and the V3-V4 region of the 16S rRNA gene was sequenced using the Illumina MiSeq(Illumina K.K., Tokyo, Japan) platform [22]. The QIIME 2 pipeline was used to analyze the sequence read data obtained from MiSeq [23]. QIIME 2, version 2021.4 (https://www.qiime2.org), was used to run the DADA2 plugin to merge forward and reverse sequences, eliminate low-quality reads, and check for chimeras to obtain amplicon sequence variants (ASVs). Representative sequences were aligned using the MAFFT plugin, and a rootless phylogenetic tree for phylogenetic diversity analysis was constructed using the FastTree plugin. A rooted phylogenetic tree was then inferred from the rootless tree, and α and β diversity analyses were performed. To determine the relative proportion of each specimen's microbiome composition and to identify each lead organism at the phylum and genus level, homology searches were done using SILVA version 138 (https://www.arb-silva.de/download/archive/), with a homology threshold of 99 % (Silva-138-99-nb-classifier). To examine the similarity of each microbiome, comparisons of α diversity (observed features, Shannon entropy) and β diversity (Bray–Curtis dissimilarity) were performed. The β diversity obtained from the analysis using QIIME 2 was subjected to principal coordinates analysis. The phylogenetic investigation of communities by reconstruction of unobserved states software (PICRUSt2) was used to predict the functional pathways in each group based on the MetaCyc pathways database [24,25].

2.3. FISH with resected specimens

FISH analysis of surgically resected pancreatic cancer specimens from 14 patients with pancreatic cancer. FISH analysis of specific microbiome bacteria, *S. mitis* and *H. biformis,* was outsourced to Chromosome Science Labo, Inc. (Sapporo, Japan). The MIT447 probe labeled with the 5′ fluorescein isothiocyanate fluorophore was used to detect *S. mitis*, and the E.bif462 probe labeled with the 5′ cyanine3 fluorophore was used to detect *H. biformis.* The FISH and 4′,6-diamidino-2-phenylindole (to stain DNA) signals were detected using a Leica CW-4000 cytogenetic workstation (Leica Microsystems K.K., Tokyo, Japan).

2.4. Statistical analyses

For the microbiome analysis, Welch's *t*-test was used to evaluate the relative proportions of microbiome composition and α diversity, while permutational multivariate analysis of variance was employed to test β diversity. For multiple comparisons, the Benjamini–Hochberg [26] adjustment was used to control the false discovery rate (FDR). All statistical tests were two-sided, and *p*-values or FDR-adjusted *p*-values (called *q*-values) <0.05 were considered statistically significant. Linear discriminant analysis (LDA) effect size (LEfSe) was applied to identify microbial taxa that differed significantly between groups, using a threshold LDA score of $\geq$3.5 for fecal samples and $\geq$2.0 for saliva samples [27].

3. Results

3.1. Decreased butyrate-producing bacteria in the gut microbiome of patients with pancreatic cancer

Fecal and salivary samples were collected from 30 patients with pancreatic cancer before treatment (the pancreatic cancer group) and from 18 healthy controls (the control group). The pancreatic cancer group had a lower BMI and lower rates of alcohol consumption and dyslipidemia than the control group (Table 1). There were no significant differences between the two groups in terms of the use of proton pump inhibitors and statins, which have been reported to affect oral and gut microbiomes [28,29]. The gut microbiome composition showed no significant differences in composition at the phylum level between the pancreatic cancer and control groups (Fig. 1A). At the genus level, however, the abundance of *Streptococcus* bacteria was significantly higher, whereas that of *Megasphaera*, *Lachnospira*, and *Holdemanella* was significantly

Table 1
Characteristics of patients with pancreatic cancer and healthy control groups.

	Pancreatic cancer	Healthy control	P value
Total samples (feces, saliva)	30, 30	18, 18	NA
Age (mean)	63.7	63.0	0.368
Sex (male)	16	12	0.364
BMI (mean)	22.0 (15.0–28.9)	24.5 (19.5–32.2)	0.00992
Smoking index >400	11	6	0.815
Alcohol	9	13	0.00448
Diabetes mellitus	5	3	1.00
Hypertention	13	10	0.412
Dyslipidemia	7	11	0.00886
PPIs	8	2	0.199
Statins	6	7	0.154
Aspirins	1	2	0.281
Laxatives	2	0	0.263
Cancer Stage (early/late)	14/16	NA	NA

lower in the gut microbiome of the pancreatic cancer group compared to the control group (Fig. 1B and C). Although there was no significant difference in α diversity of gut bacterial microbiome (Fig. 2A), an examination of β diversity showed that the gut microbiome differs significantly between the pancreatic cancer and control groups (Fig. 2B).

To investigate the metagenomic changes brought about by alterations in the bacterial microbiome of the pancreatic cancer and control groups, an analysis of MetaCyc pathways using PICRUSt2 was performed. The analysis showed that the generation of precursor metabolites and energy was significantly upregulated, while glycan pathways were significantly downregulated in the pancreatic cancer group compared to the control group (Fig. 2C).

3.2. Increase in Streptococcus in the oral microbiome of patients with pancreatic cancer

Next, we analyzed oral bacteria in both the pancreatic cancer and control groups. The analysis revealed that *Firmicutes* were more abundant at the phylum level, whereas *Proteobacteria* were significantly less abundant in the pancreatic cancer group than in the control group (Fig. 3A). At the genus level, *Streptococcus* abundance was significantly higher in the pancreatic cancer group than in the control group, whereas *Neisseria* abundance was significantly lower in the pancreatic cancer group (Fig. 3B and C). Although there was no significant difference in α diversity in the oral microbiome (Fig. 4A), the β diversity showed that the oral microbiome differs significantly between the two groups, which was consistent with the results of the gut microbiota (Fig. 4B). Analysis of MetaCyc pathways using PICRUSt2 indicated significantly differences in many pathways between the two groups, such as the upregulation of nucleoside and nucleotide biosynthesis pathways and the downregulation of fatty acid and lipid biosynthesis pathways in the pancreatic cancer group compared to the control group (Fig. 4C).

3.3. Presence of certain gut bacteria in pancreatic cancer tissues

To determine whether the bacteria detected in the gut of the pancreatic cancer group were also present in their cancer tissues, we performed microbiome FISH analysis using surgically resected pancreatic cancer specimens from 14 patients. Microbiome FISH analysis was conducted on *S. mitis*, one of the major *Streptococcus* species present in the saliva [30,31], and *H. biformis*, a representative species of the genus *Holdemanella*, which showed significant differences in the oral and gut microbiome between the pancreatic cancer and control groups in this study. *S. mitis* was detected in one pancreatic cancer sample and *H. biformis* was detected in two samples (Fig. 5A and B). These results suggest that the gut bacteria detected in patients with pancreatic cancer are also present in pancreatic cancer tissues.

4. Discussion

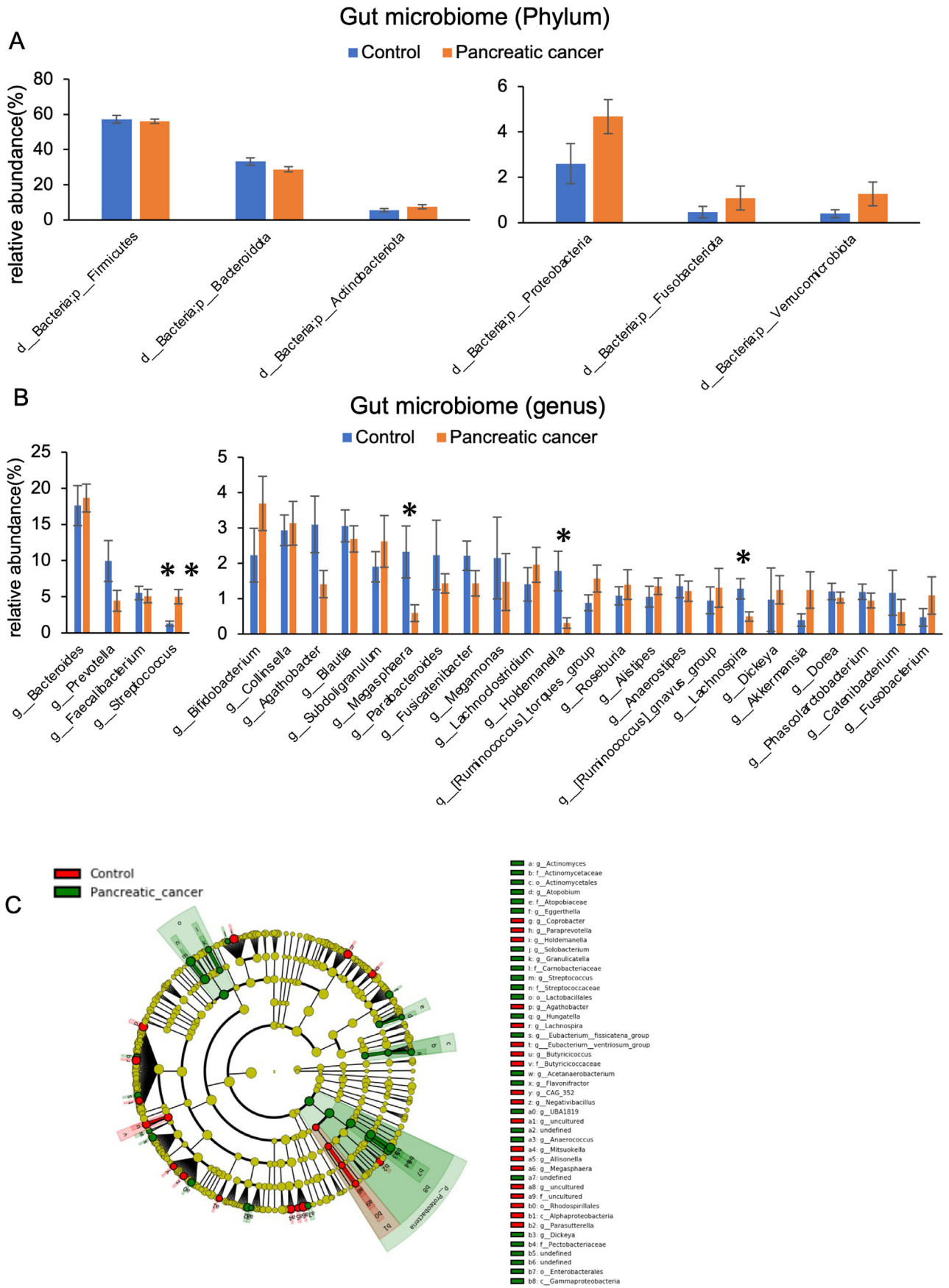
Studies exploring the association between the gut and oral microbiomes and pancreatic cancer have revealed compelling links between dysbiosis and the disease. The diagnostic implications of dysbiosis in pancreatic cancer are of paramount importance. Recent studies have investigated the potential of microbial markers for early detection and risk stratification of pancreatic cancer [32]. It has been suggested that the gut microbiome may help predict the prognosis after treatment [14]. Therapeutic interventions aimed at modulating the microbiome show promise for managing pancreatic cancer [14,33]. However, microbiomes vary among different racial groups. Therefore, the purpose of our study was to clarify the characteristics of microbiome of Japanese patients with pancreatic cancer.

It is well known that microbiome characteristics differ between oral cavity and gut. A recent study revealed that some bacteria such as *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* in salivary microbiomes are associated with higher risk of pancreatic cancer [34], however, these bacteria are not usually found in fecal microbiome. This suggests that the oral and gut microbiome related to pancreatic cancer should be investigated separately. Therefore, to better understand the potential risk of oral and gut microbiome in pancreatic cancer, we investigated both oral and gut microbiome.

In our study, Japanese patients with pancreatic cancer have different microbiome characteristics from Western cohort, in which *Proteobacteria, Actinobacteria, Fusobacteria, and Verrucomicrobia* are enriched in feces of patients with pancreatic cancer [13], whereas *Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans* are enriched in salivary microbiome of patients with pancreatic cancer [34]. Our results suggested that the microbiomes differ among races in patients with pancreatic cancer.

In this study, we demonstrated that the oral and gut microbiomes of patients with pancreatic cancer differ from those of healthy controls in Japan. The diversity of the oral and gut microbiomes in the pancreatic cancer group was reduced compared to the control group in the Japanese population, consistent with previous reports from Western countries. We also observed an increase in the genus *Streptococcus* in the oral and gut microbiome of the pancreatic cancer group, which aligns with a previous report [18], and a decrease in a few kinds of bacteria such as *Megasphaera*, *Lachnospira*, and *Holdemanella,* those are known to produce butyrate, a short-chain fatty acid (SCFA) [35–37] in the feces of the pancreatic cancer group compared to the control group in the Japanese population. These results indicate that the increase in the genus *Streptococcus* in the oral microbiome and the decrease in butyrate-producing bacteria in gut microbiome may be involved in the pathogenesis of pancreatic cancer.

Streptococcus spp. and *Megasphaera* spp. have been reported to be related to pancreatic cancer [18]. Moreover, *Megasphaera* spp. has been reported to be significantly associated with longer survival in patients with pancreatic cancer [33]. These results are consistent with our cohort, and thus, these microbiomes are considered to be characteristic of pancreatic cancer. On the other hand, *H. biformis* has been reported to be related to colorectal cancer [38]. In addition, it has been reported that patients with chronic pancreatitis or IPMN have similar microbiomes [18] including *Streptococcus* spp. Summarily, our results together with the previous reports suggest that the microbiomes in the oral cavity


A
Gut microbiome (Phylum)
Control
Pancreatic cancer
relative abundance(%)
d__Bacteria;p__Firmicutes
d__Bacteria;p__Bacteroidota
d__Bacteria;p__Actinobacteriota
d__Bacteria;p__Proteobacteria
d__Bacteria;p__Fusobacteriota
d__Bacteria;p__Verrucomicrobiota
B
Gut microbiome (genus)
Control
Pancreatic cancer
relative abundance(%)
g__Bacteroides
g__Prevotella
g__Faecalibacterium
g__Streptococcus
g__Bifidobacterium
g__Collinsella
g__Agathobacter
g__Blautia
g__Subdoligranulum
g__Megasphaera
g__Parabacteroides
g__Fusicatenibacter
g__Megamonas
g__Lachnoclostridium
g__Holdemanella
g__[Ruminococcus]_torques_group
g__Roseburia
g__Alistipes
g__Anaerostipes
g__[Ruminococcus]_gnavus_group
g__Lachnospira
g__Dickeya
g__Akkermansia
g__Dorea
g__Phascolarctobacterium
g__Catenibacterium
g__Fusobacterium
C
Control
Pancreatic_cancer
a: g__Actinomyces
b: f__Actinomycetaceae
c: o__Actinomycetales
d: g__Atopobium
e: f__Atopobiaceae
f: g__Eggerthella
g: g__Coprobacter
h: g__Paraprevotella
i: g__Holdemanella
j: g__Solobacterium
k: g__Granulicatella
l: f__Carnobacteriaceae
m: g__Streptococcus
n: f__Streptococcaceae
o: o__Lactobacillales
p: g__Agathobacter
q: g__Hungatella
r: g__Lachnospira
s: g__Eubacterium__fissicatena_group
t: g__Eubacterium__ventriosum_group
u: g__Butyricicoccus
v: f__Butyricicoccaceae
w: g__Acetanaerobacterium
x: g__Flavonifractor
y: g__CAG_352
z: g__Negativibacillus
a0: g__UBA1819
a1: g__uncultured
a2: undefined
a3: g__Anaerococcus
a4: g__Mitsuokella
a5: g__Allisonella
a6: g__Megasphaera
a7: undefined
a8: g__uncultured
a9: f__uncultured
b0: o__Rhodospirillales
b1: c__Alphaproteobacteria
b2: g__Parasutterella
b3: g__Dickeya
b4: f__Pectobacteriaceae
b5: undefined
b6: undefined
b7: o__Enterobacterales
b8: c__Gammaproteobacteria

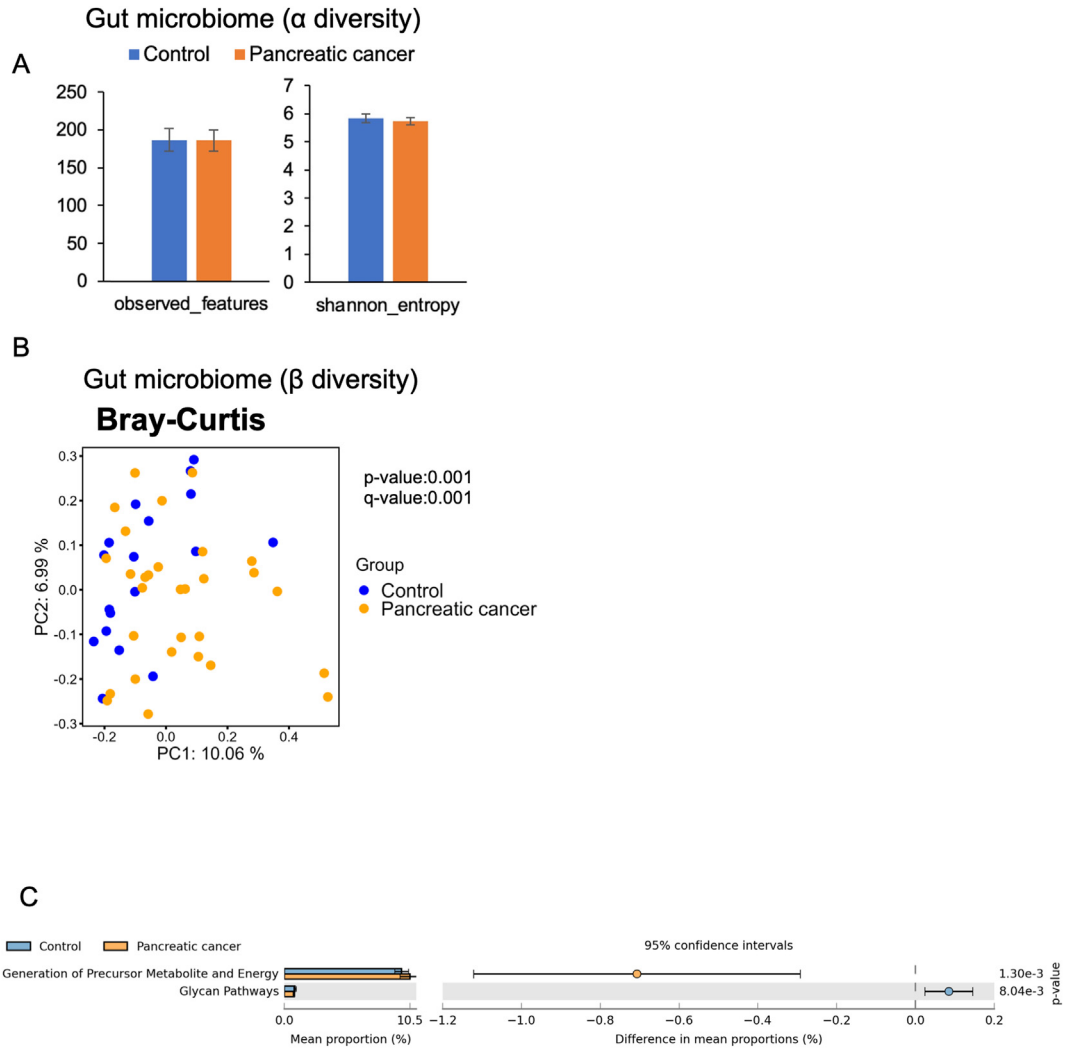


Fig. 2. Microbial diversity and MetaCyc pathways analysis of the gut microbiome of patients with pancreatic cancer and healthy controls.
(A) α diversity of the gut microbiome of the pancreatic cancer and healthy control groups. (B) β diversity of the gut microbiome of the pancreatic cancer and healthy control groups. (C) MetaCyc pathway analysis of the gut microbiome of the pancreatic cancer and healthy control groups.

and gut in patients with pancreatic cancer are not specific for pancreatic cancer. Given that the microbiome of patients with chronic pancreatitis or IPMN and that with pancreatic cancer have similarity, although we did not analyze them in our study, we assume that some kinds of microbiomes contribute to the development of pancreatic cancer.

It remains unknown whether an increase in the genus *Streptococcus* in the oral and gut bacteria and a decrease in some SCFA-producing gut bacteria in patients with pancreatic cancer could contribute to the initiation, progression, and resistance to chemotherapy. Given that *Streptococcus* spp. was observed in patients with IPMN or chronic pancreatitis [18], we speculate *Streptococcus* spp. works in a tumor-promotive manner in pancreatic cancer, although its mechanism has not been elucidated. Recently, *H. biformis* has been reported to suppress colorectal cancer progression by producing SCFA [38]. SCFA could inhibit calcineurin and NFATc3 activation, thereby suppress cancer proliferation in colorectal cancer [38]. Therefore, we speculate that the reduction of *H. biformis,* which has been reported to work in a tumor-suppressive manner [38], may contribute to pancreatic cancer progression. Moreover, most of the pathways that we have found in an analysis of MetaCyc pathways using PICRUSt2 were related to metabolic functions such as the glycolytic system and the TCA cycle. Although we could not find direct association between these pathways and tumor development in this study, this results suggests that microbiome can have an influence on the metabolic function of the host. This is consistent with the finding that a reduction in SCFA-producing bacteria such as *H. biofilms* was found in the microbiome of patients with pancreatic cancer. Future studies are required to clarify the functional roles of these bacteria in pancreatic cancer pathogenesis and resistance to chemotherapy.

In this study, we showed that *S. mitis* and *H. biformis,* which were increased and decreased in the gut microbiome of the pancreatic cancer group compared to the control group,

Fig. 1. Comparison of the gut microbiome of patients with pancreatic cancer and healthy controls.
(A) Comparison of the gut microbiome at the phylum level between the pancreatic cancer and healthy control groups. (B) Comparison of the gut microbiome at the genus level between the pancreatic cancer and healthy control groups. (C) Taxonomic cladogram of the gut microbiome of the pancreatic cancer and healthy control groups using linear discriminant analysis effect size (LEfSe).

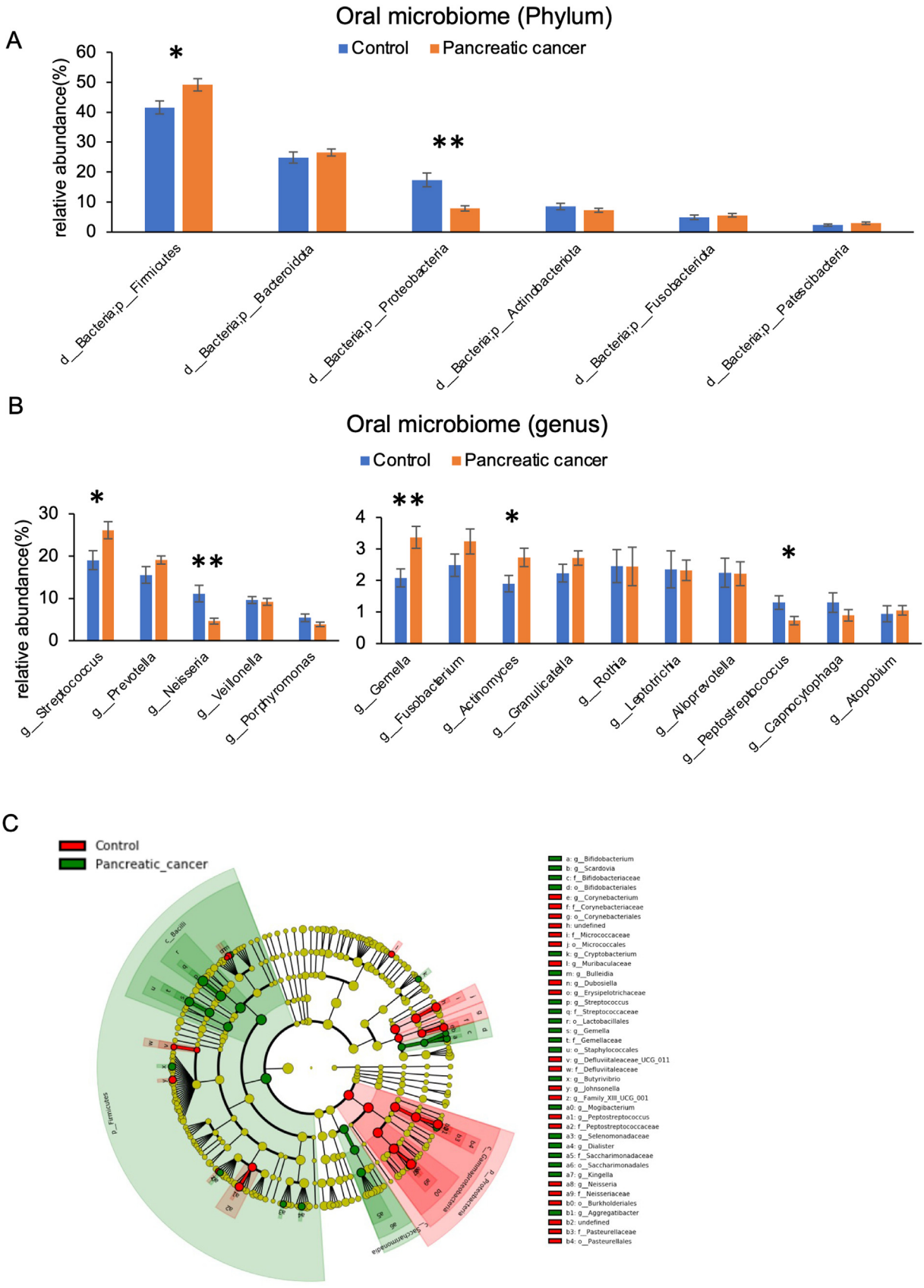


Fig. 3. Comparison of the oral microbiome of patients with pancreatic cancer and healthy controls.
(A) Comparison of the oral microbiome at the phylum level of patients with pancreatic cancer and healthy controls. (B) Comparison of the oral microbiome at the genus level of patients with pancreatic cancer and healthy controls. (C) Taxonomic cladogram of the oral microbiome of patients with pancreatic cancer and healthy controls using LEfSe.

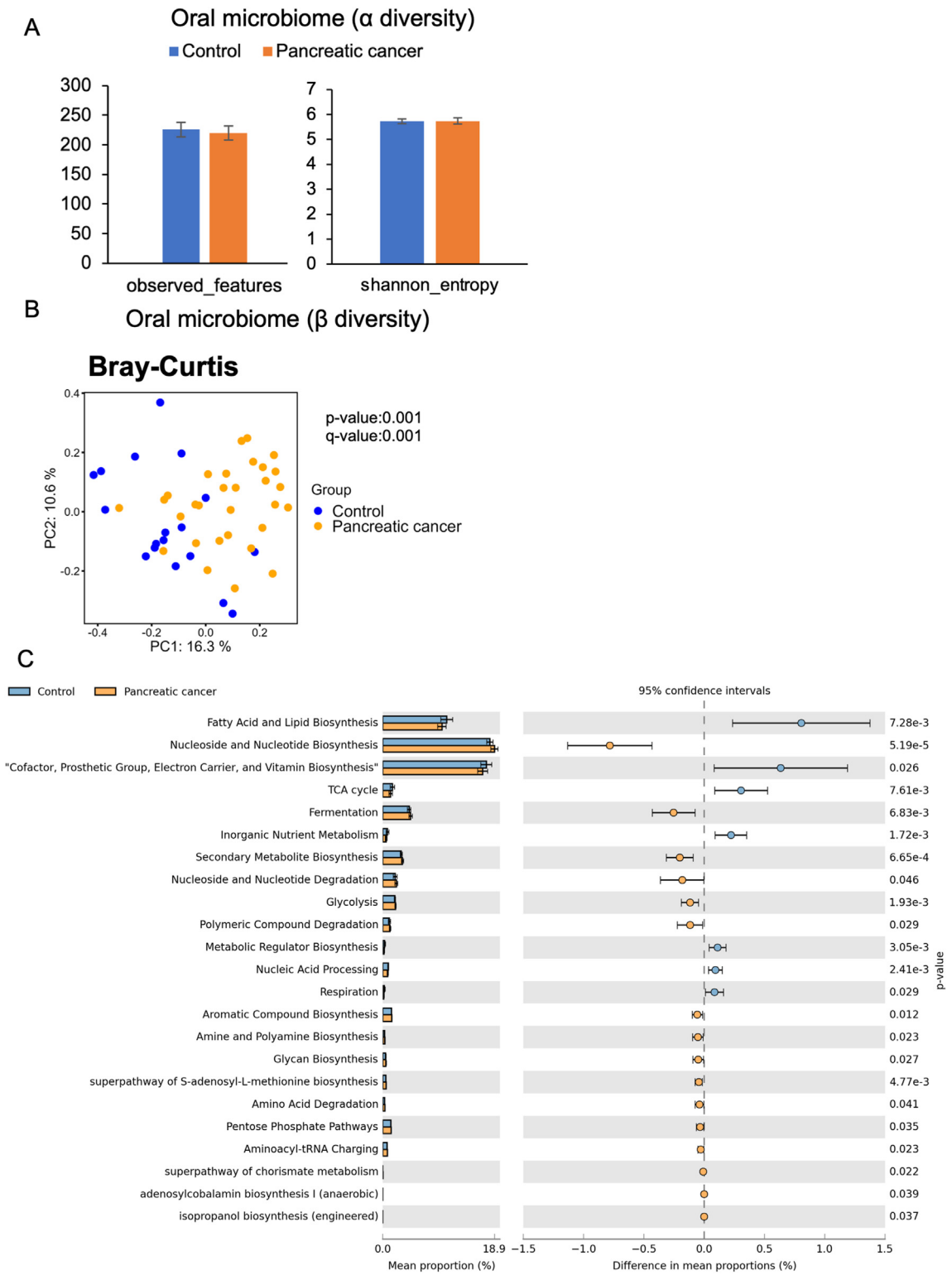


Fig. 4. Diversity and MetaCyc pathway analysis of the oral microbiome of patients with pancreatic cancer and healthy controls.
(A) α diversity of the oral microbiome of the pancreatic cancer and healthy control groups. (B) β diversity of the oral microbiome of the pancreatic cancer and healthy control groups. (C) MetaCyc pathway analysis of the oral microbiome of the pancreatic cancer and healthy control groups.

respectively, were present in a few human pancreatic cancer tissues, suggesting that it is rare for these bacteria to migrate into pancreatic tissue. Although present in a small number of cases, the detection of these bacteria in human pancreatic tissue suggests their involvement in the carcinogenesis and progression of pancreatic cancer. The exact mechanisms underlying the migration of these microbes to pancreatic cancer tissues have not been fully elucidated. However, previous studies have proposed several

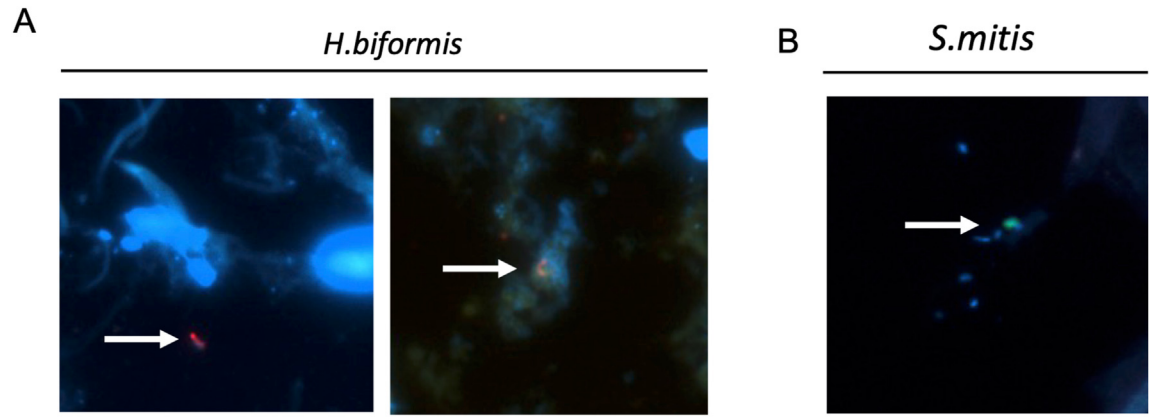


Fig. 5. Fluorescent in situ hybridization (FISH) to detect *Holdemanella biformis* and *Streptococcus mitis* in human pancreatic cancer tissues.
(A) FISH to detect *H. biformis* in pancreatic cancer tissues. (B) FISH to detect *S. mitis* in pancreatic cancer tissues.

plausible explanations, including hematogenous dissemination, lymphatic system involvement, direct extension from adjacent tissues, immune cell-mediated transport, and biliary tract involvement. These mechanisms are speculative, and the specific pathways of microbial translocation require further investigation. Additionally, factors such as the tumor microenvironment, local immune responses, and specific microbial species involved may influence migratory processes.

Our study has several limitations. The sample size is relatively small. The detection rate of microbiomes in human pancreatic specimens is low. NGS studies would be better for the confirmation of the presence of bacteria in pancreatic cancer tissue, however, we performed FISH studies targeting the bacteria because it was considered that only a very small number of bacteria was present in the pancreatic tissue and that they might not be detectable by NGS. The role of reduced SCFA in pancreatic cancer progression is still unknown. However, our study is significant in that it reveals a relationship between SCFA-producing bacteria and pancreatic cancer.

Recent studies have reported that patients with chronic pancreatitis have gut microbiota dysbiosis [39]. Chronic pancreatitis is a risk factor for the development of pancreatic cancer. Therefore, if bacteria such as *S. mitis* and *H. biformis* are present in patients with chronic pancreatitis, it is more likely that they contribute to the development of pancreatic cancer. Future study is warranted to investigate the microbiome of patients with chronic pancreatitis. Moreover, given that a recent study revealed that *Clostdirium butyricum*, a butyrate-producing probiotic, inhibits intestinal tumor development [40], it is tempting to see whether administration of SCFA-producing probiotics to patients with pancreatic cancer inhibits the progression of pancreatic cancer.

In conclusion, we demonstrated that the gut and oral microbiomes differ significantly between patients with pancreatic cancer and healthy controls in Japan. We also observed an increase in the genus *Streptococcus* in the oral and gut microbiome and a decrease in several SCFA-producing genera in the feces of the pancreatic cancer group compared to the control group. Further research in this area will contribute to a more comprehensive understanding of the complex interactions between the microbiome and pancreatic cancer.

Author contribution

M. Sono and K.Iimori contributed equally to this work. **M. Sono**: Conceptualization, data curation, formal analysis, investigation, visualization, methodology, writing-original draft. **K. Iimori**: Conceptualization, data curation, formal analysis, visualization, writing-original draft. **M. Nagao**: Investigation, methodology. **S.Ogawa**: Investigation, methodology, writing-review and editing. **T. Maruno**: Investigation, methodology. **Y. Nakanishi**: Funding acquisition, investigation, methodology. **T. Anazawa**: Resources. **K. Nagai**: Resources. **T. Masui**: Resources. **H. Mori**: Formal analysis, methodology, writing-review and editing. **K. Hosomi**: Formal analysis, methodology, supervision, writing-review and editing. **J. Kunisawa**: Formal analysis, methodology. **H. Yokota**: Data curation, formal analysis, visualization. **Y. Tanaka**: Data curation, formal analysis, visualization. **H. Ohno**: Data curation, formal analysis, visualization. **E. Hatano**: Resources. **A. Fukuda**: Conceptualization, supervision, funding acquisition, methodology, project administration, writing-review and editing. **H. Seno**: Supervision, funding acquisition.

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Ethics approval and consent to participate

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Kyoto University (Protocol no. R1844-2). Written informed consent was obtained from all study participants for microbiome analysis. For FISH with resected specimens, informed consent was obtained in the form of opt-out on the website.

Declaration of conflict of interest

All authors except Haruka Yokota, Yoshiki Tanaka, Hiroshi Ohno

declare no conflict of interest. They are employed by Biofermin Pharmaceutical Co., Ltd.

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