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Original Article

Analysis of oral microbiota in Japanese oral cancer patients using 16S rRNA sequencing

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Objectives: It is important to determine the cause of increasing oral cancer occurrence and mortality rates in Japan, because the mortality rate has recently decreased in other developed countries. The impact of microbiota in carcinogenesis, especially in the digestive tract has been reported. This study aimed to clarify the relationship between oral cancer and oral microbiota in Japanese patients.
Methods: DNA was extracted from salivary samples of 60 oral cancer patients and 80 non-cancer individuals as controls. We performed metagenomic analysis using 16S rRNA amplicon sequencing. Statistical analysis in this study was performed using R (version 3.5.0).
Results: Oral cancer patients showed higher α-diversity compared to the control group, and the β-diversity between the two groups differed significantly. Further, there was a significant difference in the abundance ratio of bacterial genera between the two groups. *Peptostreptococcus*, *Fusobacterium*, *Alloprevotella*, and *Capnocytophaga* were more abundant in the cancer group compared to the control, whereas *Rothia* and *Haemophilus* were less abundant ($p < 0.01$). A negative correlation in the microbiota composition was confirmed between the operational taxonomic units (OTU) of genus *Rothia* and T-stage progression using the TNM classification method. We performed logistic regression analysis to investigate the impact factor for the oral cancer group, and the result showed that Chao 1 index and sex are statistically significant variables.
Conclusions: In this study, we observed an increased bacterial diversity in oral cancer patients and found distribution changes for some bacteria.

1. Introduction

In recent years, the occurrence of oral cancer has increased in developed countries, with an estimated 7000 new cases and 3000 deaths occurring annually in Japan alone. Oral cancer, which accounts for only 2–3% of all cancers, most commonly involves the tongue, and is associated with an age-adjusted male to female ratio of 2:1. Approximately 90% of the pathological types of cancer are oral squamous cell carcinoma (OSCC). The choices for oral cancer therapy comprise surgical treatment, chemotherapy, and radiation. The five-year survival rate of oral cancer is said to be 60–80%; however, early detection of cancer increases the survival rate to approximately 90% [1,2]. The etiology of oral cancer is multifactorial. Chemical irritants, such as cigarette smoke and alcohol, are associated with the risk of oral cancer, along with physical irritation by poor dental prosthesis or other factors such as a human

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papillomavirus infection. Thus, excessive drinking and smoking increase the risk of oral cancer. Chronic periodontitis has also been reported as a risk factor for oral cancer [3,4].

A relationship between gut microbiota and tumorigenesis in the digestive region has been reported previously. For example, *Helicobacter pylo*ri causes gastric cancer [5]. *Salmonella typhi* and *Fusobacterium* are associated with gallbladder and colon cancer, respectively [6,7]. Further, a possible association between oral microbiota and colorectal cancer has been reported. Several oral taxa such as *Streptococcus* and *Prevotella* were found to be abundant in colorectal cancer patients compared to a healthy control group [8]. Further, bacterial and fungal microbiota have been reported in association with oral cancer. Approximately 700 bacterial species and 10 *Candida* fungal species have been detected in the human oral cavity. However, there is no clarification about their association with the risk of oral cancer. Some studies have employed next-generation sequencing (NGS) to assess the oral microbiota associated with oral cancer [9–11]. However, the number of subjects was too small, and samples of cancer lesions and healthy sites from the same subject were used in these studies. Therefore, the credibility of the findings is unclear. Most of these studies have been reported in USA and in Asian countries except Japan with national and regional differences in the oral microbiota composition [12]. However, there is no large-scale report regarding the comparison of oral microbiota between oral cancer patients and non-cancer control groups in Japan.

In this study, we report the oral microbiota of Japanese oral cancer patients compared with that in non-cancer subjects, using 16S rRNA amplicon sequencing of saliva samples. This study aimed to clarify the relationship between oral microbiota and oral cancer occurrence in Japan.

2. Materials and methods

2.1. Sample collection

In total, 140 salivary samples taken from patients between 2016 and 2018 were included in this study; this was approved by the Research Ethics Committee at Southern TOHOKU General Hospital (Approval number: 216–3), Tsurumi University (Approval number: 1523) and the National Institute of Biomedical Innovation, Health and Nutrition (Approval number: 167). Prior to sample collection, written informed consent was obtained from all patients. Patients recruited from the Southern TOHOKU General Hospital were classified into two groups: control (80 noncancer individuals) and OSCC (60 patients). The control group was defined as individuals without any diagnosed mucosal diseases and other cancers and these individuals were over the age of 40. The diagnosis of a healthy oral cavity was made after a thorough clinical examination. All diagnoses of OSCC were confirmed by biopsy and pathological findings. Participants who did not follow the instructions or had low saliva secretion capacity were excluded. Participants who were undergoing chemo radiotherapy or had received antibiotics treatment within 28 days were excluded. The participants were asked to chew gum for 5 min and stimulated saliva samples were collected into sterile plastic tubes. The samples were stored at −80 °C until use. The participants completed a questionnaire regarding sex, age, drinking, and smoking habits as well as denture use.

2.2. DNA extraction

DNA was extracted according to a previously described protocol [13]. Homogenization of the suspended salivary samples (200 μl) was performed using beads with 300 μl lysis buffer (No. 10, Kurabo Industries Ltd., Osaka, Japan) and 0.5 g of 0.1-mm glass beads in 2-ml vials. The mixture was mechanically disrupted using a Cell destroyer PS1000 (Bio Medical Science, Tokyo, Japan) at 4260 rpm for 50 s at room temperature (20–25 °C). All samples were centrifuged at 12,000×*g* for 5 min at room temperature. The supernatant was collected and mixed with 150 μl lysis buffer and 150 μl proteinase K buffer (No. 2, Kurabo Industries Ltd) containing 0.4 mg/ml proteinase K. DNA was extracted using a Gene Prep Star PI-80X device (Kurabo Industries Ltd). The extracted DNA was determined using a NanoDrop Spectrophotometer ND-1000 (Thermo Fisher Scientific Inc., DE, USA), and the samples were stored at −30 °C until use.

2.3. 16S rRNA sequencing

16S rRNA sequencing was performed as described previously [13]. The V3–V4 region of the 16S rRNA gene was amplified from salivary DNA samples using previously published primers. The reaction process was performed at 95 °C for 3 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 min, with a final extension at 68 °C for 5 min. PCR products were purified with 20 μl of Agencourt AMPure XP (Beckman Coulter, Inc., CA, USA) in accordance with the manufacturer's protocol and eluted into 50 μl of 10 mM Tris–HCl, pH 8.5. For DNA library preparation, Illumina adapters were attached to the PCR products using Illumina MiSeq Nextera kit set A (Illumina Inc., CA, USA). 16S rRNA gene sequencing of the PCR products was performed using Illumina MiSeq (Illumina) in accordance with the manufacturer's instructions.

2.4. Bioinformatics analysis

FASTQ files were obtained after Illumina pair-end 16S rRNA gene amplicon sequencing, and the operational taxonomic units (OTUs) classification and diversity analyses were performed using QIIME version 1.9.1 [14] according to previously described methods [15]. The sequences were clustered into OTUs by an open-reference OTU picking process against the SILVA 128 reference database [16] at 97% similarity using USEARCH [17]. The OTUs were classified taxonomically up to genus level using the SILVA 128 reference database [16].

2.5. Statistical analysis

The resulting data were exported as BIOM files and imported to R (version 3.5.0). Diversity analysis was performed using the "phyloseq" R-package [18]. α-diversity indexes (OTU observed, Chao 1 Index, Shannon Index, and Simpson Index) were calculated by the *estimate_richness* function and the β-diversity index, calculated by unweighted UniFrac distance and weighted UniFrac distance, was generated using the *unifrac* function in the "phyloseq" R-package. Oral bacterial community structure similarity of each sample was calculated using principal coordinate analysis (*dudi.pco* function in "ade4" R-package). The dominant bacteria from phylum to genus level were defined as the mean of the distribution of bacterial composition with at least 0.05%. Student's t-test was used to compare the dominant oral bacterial community (larger than 1%) between the OSCC and controls (*t.test* function in "stats" R-package). To compare the relative abundances of taxa between OSCC samples of different T-stage or N-stage, we used the Wilcoxon rank sum test (*wilcox.test* function in "stats" R-package) and Pearson correlation analysis (*cor* function in "stats" R-package), respectively. Logistic regression analysis by the stepwise-selected model was employed to identify the impact factor for cancer incidence (*glm* function and *step* function in "stats" R-package).

Statistical analysis in this study was performed using R (version 3.5.0). All statistical tests were two-sided, with a P-value < 0.05

considered significant. All graphs were created using R package "ggplot2" [19].

2.6. Data availability

The sequencing data from this study have been deposited in the NCBI Sequence Read Archive under accession numbers SRR8675978 – SRR8676117.

3. Results

To reveal the differences in the oral microbiota associated with oral cancer, we collected the salivary samples from 60 OSCC patients (10 females, 50 males) and 80 control subjects (43 females, 37 males). There was no difference in the average age (63.7 and 65.1 years) between OSCC patients and control subjects.

We obtained a set of 11, 748, 091 raw reads upon sequencing the 16S rRNA V3–V4 region from the DNA samples. The number of sequences per sample ranged from 16,435 to 146,411 and had an average of 83,915 sequences per sample.

After sorting, unique representative sequences were classified into 14,057 operational taxonomic units (OTU) at a 97% similarity level, from which 273 genera were detected. After picking 10,000 reads per sample randomly, unique representative sequences were classified into 7647 OTUs, from which 225 genera were detected.

3.1. Diversity

Different indexes (OTU observed, Chao 1, Shannon, and Simpson) were employed to estimate the α-diversity of the bacterial community. As revealed by the OTU observed and Chao 1 index, the diversity of the bacterial community in the OSCC samples was significantly higher compared with that in the control samples ($p < 0.05$). No difference was observed with other diversity indexes (Shannon, Simpson) (Fig. 1 A – D).

To compare whether the overall bacterial taxa composition was different between the OSCC samples and controls, we used principal coordinates analysis (PCoA) on weighted and unweighted Unifrac distances.

We observed statistically significant differences in both weighted ($p = 0.009$) and unweighted ($p = 0.003$) Unifrac distances between the OSCC samples and controls (Fig. 1 E, F).

3.2. Relative abundance

The bacterial communities in the OSCC samples and controls were analyzed at the genus level with a relative abundance lower than 0.01%. The number of genera identified in this study was 85. At the genus level, *Neisseria*, *Veillonella*, *Streptococcus*, *Prevotella 7*, and *Haemophilus* were the five most abundant genera. In the OSCC samples, there was a dominance of *Neisseria* (13.56 ± 1.54%), *Veillonella* (13.74 ± 1.31%), *Streptococcus* (10.14 ± 0.92%), *Prevotella 7* (10.19 ± 0.87%), and *Haemophilus* (6.49 ± 0.83%). In the controls, *Neisseria* (16.47 ± 1.40%), *Veillonella* (14.06 ± 0.98%), *Streptococcus* (12.30 ± 0.88%), *Prevotella 7* (11.46 ± 0.95%), and *Haemophilus* (9.40 ± 0.81%) were dominant. We observed no significant difference in the abundance of the top three bacterial genera between the OSCC samples and controls (Fig. 2).

A significant level of abundance was observed for *Peptostreptococcus*, *Fusobacterium*, *Alloprevotella*, and *Capnocytophaga* ($p < 0.05$) in OSCC samples relative to the controls (Table 1, Fig. 3 A – D). In contrast, we observed a lower abundance of *Rothia* and *Haemophilus* ($p < 0.05$) in the OSCC samples relative to the controls (Table 1, Fig. 3 E, F).

3.2.1. Relationship between bacteria and other factors

We obtained information regarding age, sex, drinking, and smoking habits as well as denture use from participants. Statistical analysis was performed to compare these items with the six bacterial genera that were identified to be significantly involved in cancer occurrence (Table 2).

In the OSCC group, we observed a significantly high abundance of *Peptostreptococcus* and a low ratio of *Haemophilus* in female subjects compared to male subjects (Fig. 4 A, B). In the OSCC group, *Haemophilus* was significantly abundant in drinkers (Fig. 4C).

3.2.2. Logistic regression analysis

To identify the impact factor for oral cancer occurrence, logistic regression analysis was performed. We conducted an analysis to predict cancer based on age, sex, smoking, drinking habits, denture use, OTU observed index, Chao 1 index, Shannon index, Simpson index, and the OTUs of the six dominant genera (*Rothia*, *Alloprevotella*, *Capnocytophaga*, *Peptostreptococcus*, *Fusobacterium*, and *Haemophilus*). We obtained independent variables by eliminating multicollinearity after a stepwise procedure. The result contained two interaction terms (sex, Chao 1 index). We obtained an odds rate of 10.85 for sex (97.5% CI) and 1.006 for Chao 1 index (97.5% CI) (Table 3).

3.2.3. TNM classification

We performed a stratified analysis to investigate the difference between tumor stage and the relative abundance of dominant taxa using the TNM (tumor-node-metastasis) classification of the UICC (International Union Against Cancer) 7th edition. The number of patients in each stage was T1: 7, T2: 16, T3: 14, T4: 22, TX: 1 and N0: 20, N1: 7, N2: 32, N3: 1. None of the patients showed metastasis.

We observed that the α-diversity of OTUs decreased with the progression of T stage. There was a negative correlation among the stages, $r = -0.329$ (Fig. 5A).

N stages were divided into two groups: N negative (N0) and N positive (N1 to 3) to investigate the presence or absence of lymph node metastasis.

We found no significant difference in the α-diversity between N negative and N positive ($p = 0.370$) (Fig. 5B).

We performed a stratified analysis to investigate the relationship between the progression of T stage and bacterial community changes. For this purpose we used bacterial genera, which previously indicated a significant difference in cancer occurrence (*Peptostreptococcus*, *Fusobacterium*, *Alloprevotella*, *Capnocytophaga*, *Rothia*, and *Haemophilus*). As a result, the abundance of the *Rothia* genus was relatively decreased with T stage progression, showing a negative correlation among the stages, $r = -0.323$ (Fig. 5C).

4. Discussion

Some studies have assessed the characteristics of the microbiota in OSCC patients and healthy controls by comparing those groups [20–23]. However, due to the small number of research subjects and lack of research in Japanese people, we have conducted the present study comparing between OSCC patients and noncancer controls using salivary samples that reflect the entire oral microbiota and performing metagenome analysis by 16S rRNA sequencing.

4.1. α-diversity and β-diversity

We analyzed the microbiota diversity and compared the constituent bacteria. α-diversity compares the differences of individuals and β-diversity compares the differences among groups. α-diversity is calculated from several diversity indexes. The

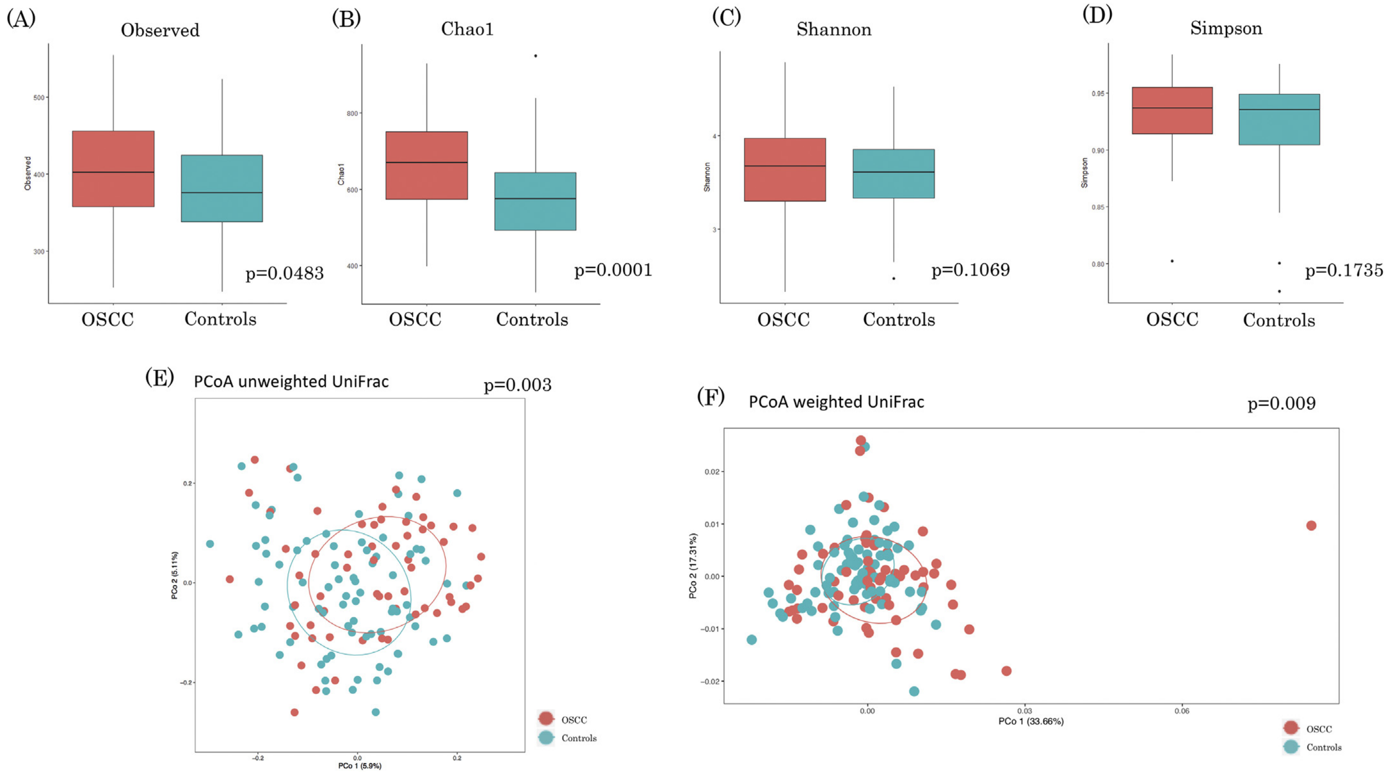

Fig. 1. Comparison of α-diversity and β-diversity in OSCC patients and controls. In Observed and Chao 1 index (A) (B), we recognized diversity abundance, but there was no significant difference between the Simpson index and Shannon index (C) (D). PCoA based on weighted UniFrac distance (E). PCoA based on unweighted UniFrac distance (F). PCoA revealed that β-diversity in OSCC was significantly different in the controls.

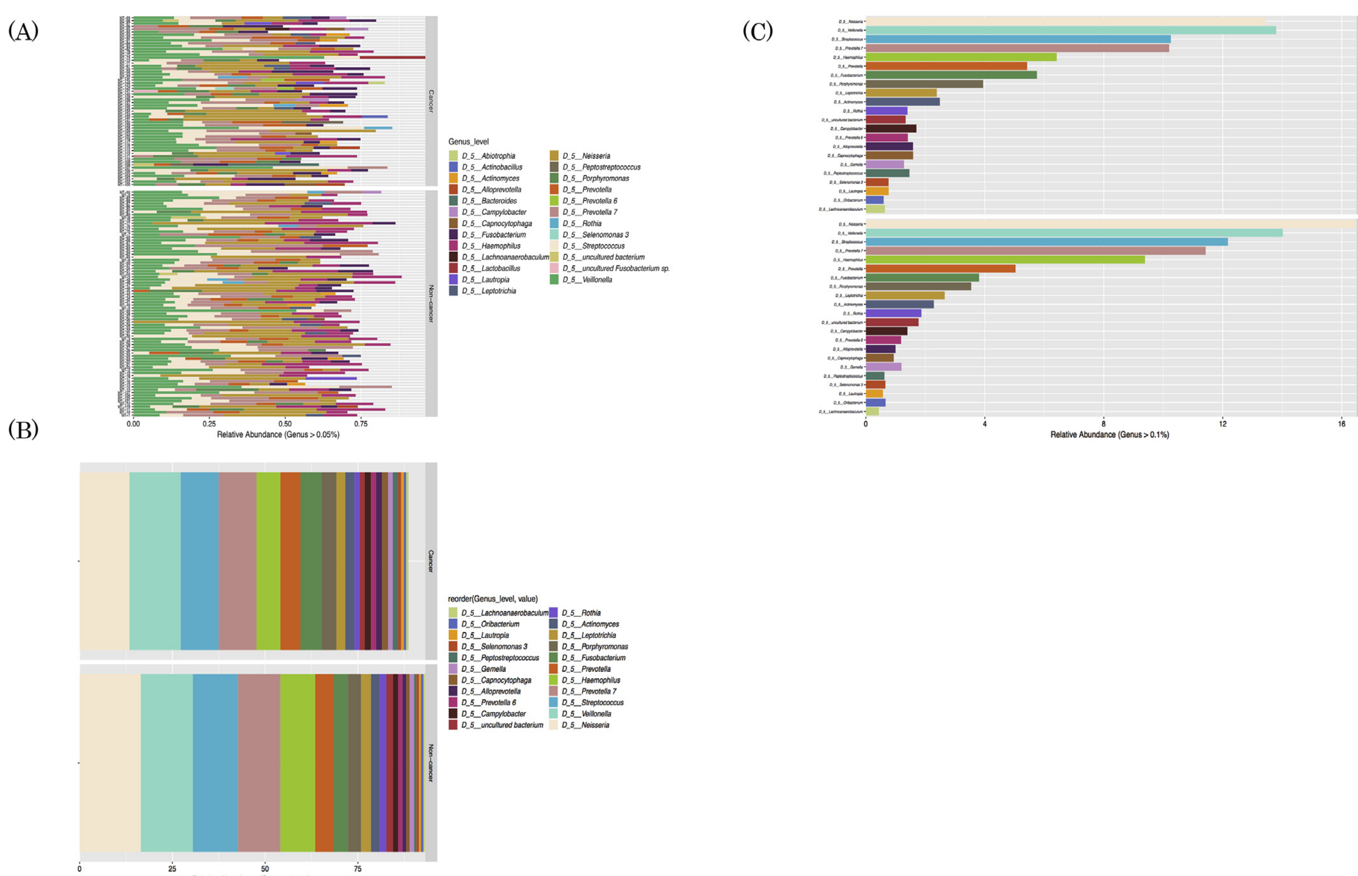

Fig. 2. Composition of bacterial communities across the samples at genus level (A) Relative abundance between individuals (B) Relative abundance between OSCC and the controls (C) Rearrangement of bacteria from the controls in descending order.

Table 1
Bacteria that showed a significant difference in OSCC patients and the controls.

	p-value
D:1::Actinobacteria_D:2::Actinobacteria_D:3::Micrococcales	0.000999
D:1::Actinobacteria_D:2::Actinobacteria_D:3::Micrococcales_D:4::Micrococcaceae	0.000999
D:1::Actinobacteria_D:2::Actinobacteria_D:3::Micrococcales_D:4::Micrococcaceae_D:5::Rothia	0.000999
D:1::Bacteroidetes_D:2::Bacteroidia_D:3::Bacteroidales_D:4::Prevotellaceae_D:5::Alloprevotella	0.009487
D:1::Bacteroidetes_D:2::Flavobacteriia	0.0221
D:1::Bacteroidetes_D:2::Flavobacteriia_D:3::Flavobacteriales	0.0221
D:1::Bacteroidetes_D:2::Flavobacteriia_D:3::Flavobacteriales_D:4::Flavobacteriaceae	0.021857
D:1::Bacteroidetes_D:2::Flavobacteriia_D:3::Flavobacteriales_D:4::Flavobacteriaceae_D:5::Capnocytophaga	0.004448
D:1::Firmicutes_D:2::Clostridia	0.013132
D:1::Firmicutes_D:2::Clostridia_D:3::Clostridiales	0.013132
D:1::Firmicutes_D:2::Clostridia_D:3::Clostridiales_D:4::Peptostreptococcaceae	0.006903
D:1::Fusobacteria_D:2::Fusobacteriia_D:3::Fusobacteriales_D:4::Fusobacteriaceae	0.027351
D:1::Fusobacteria_D:2::Fusobacteriia_D:3::Fusobacteriales_D:4::Fusobacteriaceae_D:5::Fusobacterium	0.027351
D:1::Proteobacteria_D:2::Gammaproteobacteria	0.017997
D:1::Proteobacteria_D:2::Gammaproteobacteria_D:3::Pasteurellales	0.017721
D:1::Proteobacteria_D:2::Gammaproteobacteria_D:3::Pasteurellales_D:4::Pasteurellaceae	0.017721
D:1::Proteobacteria_D:2::Gammaproteobacteria_D:3::Pasteurellales_D:4::Pasteurellaceae_D:5::Haemophilus	0.011794

D:1 phylum, D:2 class, D:3 order, D:4 family, D5: genus.

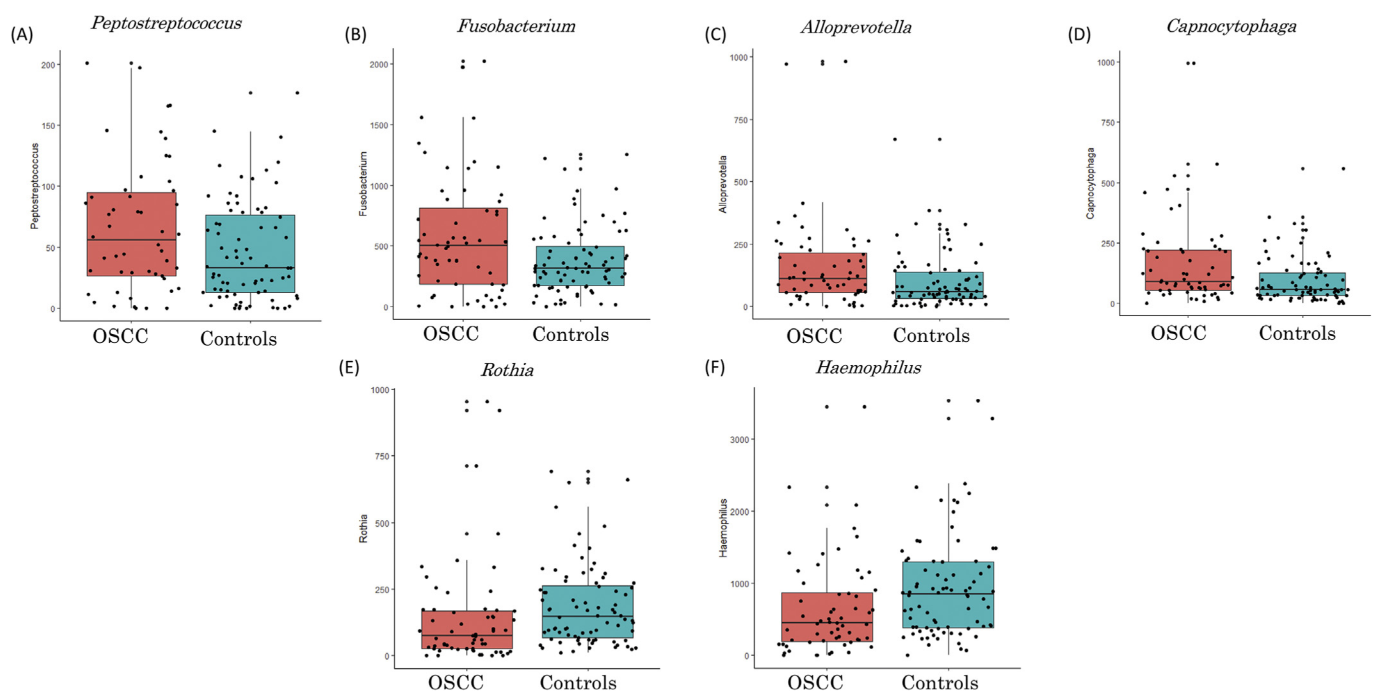

Fig. 3. Comparison of relative abundance of the genera between OSCC and the controls. *Peptostreptococcus, Fusobacterium, Alloprevotella*, and *Capnocytophaga* were frequently observed in OSCC patients (A)–(D), whereas, *Rothia* and *Haemophilus* were less (E) (F). Each black dot shows the individual OTUs.

observed index counts the number of OTUs, comparing the diversity with the relative number of bacteria. Chao 1 index compares diversity using the abundance data of bacterial types; Shannon index and Simpson index compare the diversity using the uniformity degree of existent bacterial numbers.

Regarding β-diversity, a phylogenetic tree was created from OTUs, and similarity was compared using phylogenetic distance.

Weighted UniFrac distance does not consider the number of reads whereas unweighted UniFrac distance does. The former reflects the presence or absence of bacterial species, and the latter reflects the composition ratio of bacteria.

In this study, α-diversity between OSCC patients and controls was observed with a significant difference in the observed and Chao 1 indexes. A significant difference could not be found for the Shannon or Simpson indexes (Fig. 1 A − D). The results indicated that more bacterial species were observed in the OSCC patients than in the controls, also including small counts of rare bacteria.

β-diversity results showed significant differences in Unifrac unweighted distance and Unifrac weighted distance (Fig. 1 E, F), confirming that the variation of diversity is different between the OSCC patients and controls. From these results, we concluded that the oral environment of OSCC patients is more complex with various kinds of bacteria.

Two previous studies compared healthy tissue samples and cancer tissue samples in the same patient and reported a significant difference in the Shannon index in one report [24], but not in the other report [11].

Regarding β-diversity, both reports state that Unifrac unweighted distance demonstrated an increase in the diversity of the microbiota in cancer tissue, which was similar to our results in that

Table 2
Relationship between bacteria and other factors that showed significant difference between OSCC patients and the controls(p-value).

	sex	smoke	drink	dentures
D:1::Actinobacteria_D:2::Actinobacteria_D:3::Micrococcales	0.881727	0.812257	0.712946	0.412272
D:1::Actinobacteria_D:2::Actinobacteria_D:3::Micrococcales_D:4::Micrococcaceae	0.881727	0.812257	0.712946	0.412272
D:1::Actinobacteria_D:2::Actinobacteria_D:3::Micrococcales_D:4::Micrococcaceae_D:5::Rothia	0.881727	0.812257	0.712946	0.412272
D:1::Bacteroidetes_D:2::Bacteroidia_D:3::Bacteroidales_D:4::Prevotellaceae_D:5::Alloprevotella	0.538589	0.122991	0.948044	0.183566
D:1::Bacteroidetes_D:2::Flavobacteriia	0.36153	0.418705	0.288321	0.816393
D:1::Bacteroidetes_D:2::Flavobacteriia_D:3::Flavobacteriales	0.36153	0.418705	0.288321	0.816393
D:1::Bacteroidetes_D:2::Flavobacteriia_D:3::Flavobacteriales_D:4::Flavobacteriaceae	0.36153	0.418705	0.288321	0.816393
D:1::Bacteroidetes_D:2::Flavobacteriia_D:3::Flavobacteriales_D:4::Flavobacteriaceae_D:5::Capnocytophaga	0.773631	0.537545	0.349628	0.775678
D:1::Firmicutes_D:2::Clostridia	0.00638	0.336654	0.241798	0.401419
D:1::Firmicutes_D:2::Clostridia_D:3::Clostridiales	0.00638	0.336654	0.241798	0.401419
D:1::Firmicutes_D:2::Clostridia_D:3::Clostridiales_D:4::Peptostreptococcaceae	0.04729	0.580612	0.31974	0.228898
D:1::Fusobacteria_D:2::Fusobacteriia_D:3::Fusobacteriales_D:4::Fusobacteriaceae	0.485039	0.386176	0.790712	0.946412
D:1::Fusobacteria_D:2::Fusobacteriia_D:3::Fusobacteriales_D:4::Fusobacteriaceae_D:5::Fusobacterium	0.485039	0.386176	0.790712	0.946412
D:1::Proteobacteria_D:2::Gammaproteobacteria	0.062083	0.33664	0.01242	0.781074
D:1::Proteobacteria_D:2::Gammaproteobacteria_D:3::Pasteurellales	0.054838	0.405012	0.01365	0.762779
D:1::Proteobacteria_D:2::Gammaproteobacteria_D:3::Pasteurellales_D:4::Pasteurellaceae	0.054838	0.405012	0.01365	0.762779
D:1::Proteobacteria_D:2::Gammaproteobacteria_D:3::Pasteurellales_D:4::Pasteurellaceae_D:5::Haemophilus	0.02938	0.564298	0.01798	0.652927

D:1 phylum, D:2 class, D:3 order, D:4 family, D5: genus.

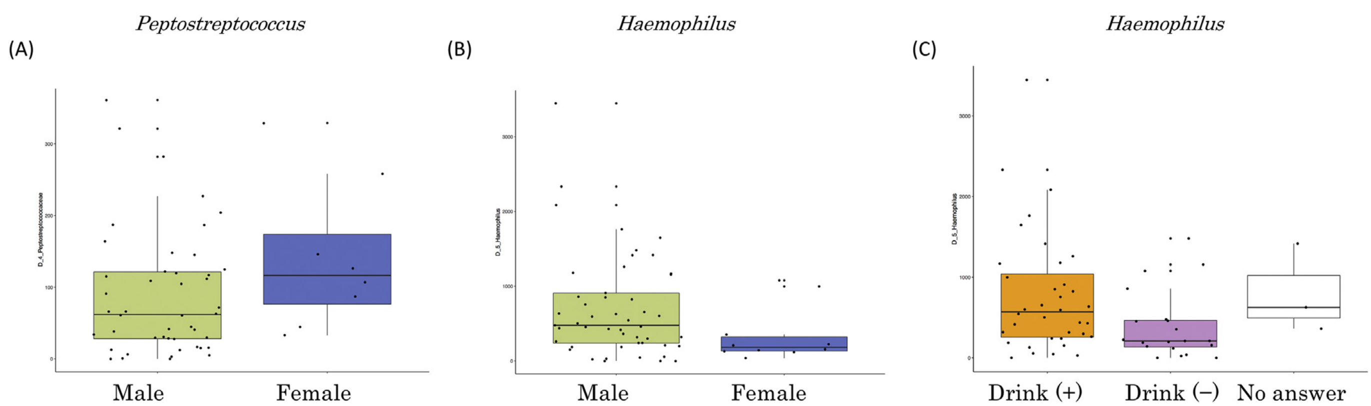

Fig. 4. Relative abundance of genera associated with impact factor and cancer incidence. *Peptostreptococcus* was frequent in females, and *Haemophilus* was frequent in males and drinkers (A)–(C). Each black dot shows the individual OTUs.

Table 3
Determination of risk factors involved in cancer incidence using logistic regression analysis.

	Estimate	p-value	Odds rate	CI (2.5%)	CI (97.5%)
(Intercept)	−8.28246	0.0004	2.529142×10^{-4}	1.810387×10^{-6}	0.01898903
Sex	2.384255	5.56×10^{-6}	10.85098	4.105171	32.79900154
Chao 1	0.006005	0.00627	1.006023	1.001851	1.01061588
Peptostreptococcaceae	0.002257	0.06608	1.00226	1.000202	1.00569097
Shannon	0.030569	0.96019	1.031041	3.092225	3.48795998

the complexity of the microbiota was increased. The results of this study also revealed the characteristics of the microbiota (Figs. 2–4 Tables 1 and 2).

4.2. Comparison of relative abundance of genera

Peptostreptococcus was abundant in OSCC patients. Genus *Fusobacterium* includes obligate anaerobic bacteria and is considered to be related to apical periodontitis and periodontal disease [25–27]. It has been consistently reported that *Peptostreptococcus* is markedly increased in OSCC samples [10,11,28]. There was also a report that *Peptostreptococcus stomatis* is involved in colorectal cancer [29].

Another report suggested that *Fusobacterium* is markedly increased in OSCC patients and is also involved in cancer progression [11]. *Fusobacterium* is known as a key bacterium forming crosslinked biofilm structures during colony formation and coexisting with other bacteria. Therefore, it is possible that the action of *Fusobacterium* may result in increased diversity in OSCC patients.

It has been reported that *Veillonella* is markedly decreased in samples of patients who have completed cancer treatment [30]. However, in our results there was no difference in the ratio between the two groups.

There was a report that the genus *Capnocytophaga* was remarkably higher in salivary samples from lung cancer patients than from healthy subjects [31]. Another report also suggested that *C. gingivalis* may be a biomarker for oral cancer [32].

In the comparison of microbiota with the TNM classification, a negative correlation was observed in progression of the T factor indicating tumor size and invasion degree. α-diversity (observed index) decreased with progression of the T factor (Fig. 5A).

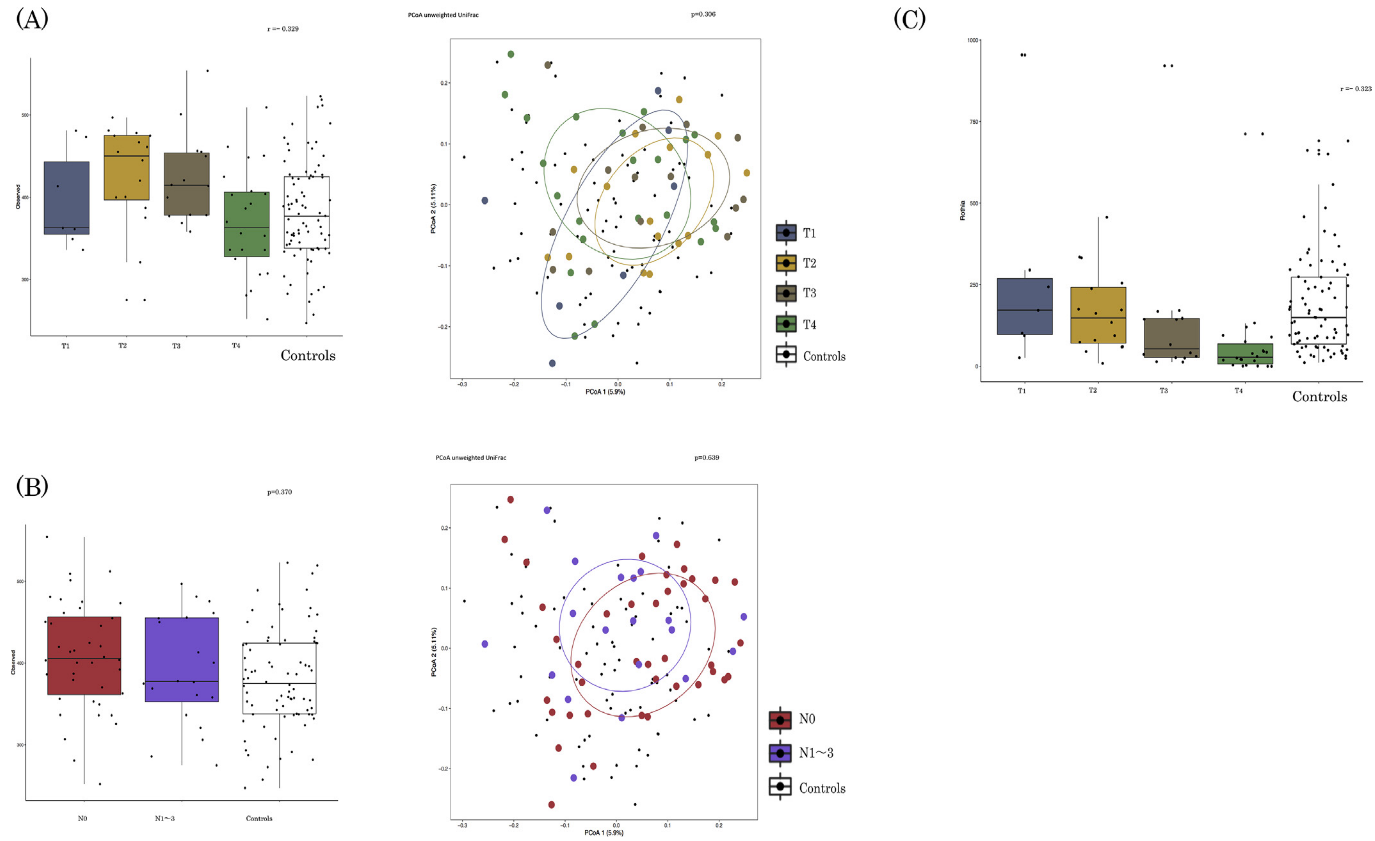

Fig. 5. Comparison of microbiota depending on the TNM-stage progression. α-diversity decreased from T2 to T4. β-diversity showed a significant correlation (A). No diversity difference was observed with N factor (B). Relative abundance of genus *Rothia* was associated with T-stage progression. *Rothia* revealed a negative correlation which decreases with progression of T factor (C). Each black dot shows the individual OTUs.

However, since α-diversity (observed index) was increased as a whole in the OSCC patients compared to the healthy controls, changes in the oxygen exposure state, the influence of exudates from necrotic tissue, and changes in the immune state can occur as the tumor increases. It is also possible that the microbiota changes.

For the N factor indicating lymph node metastasis, progressing α-diversity (observed index) was not observed (Fig. 5B). Regarding the presence or absence of lymph node metastasis, a relationship with tumor size could not be clarified. The participants of this study showed lymph node metastasis at T4 and also at T1, due to which the relevance could not be confirmed. The nature of the tumor surface tissue influences the changes in microbiota. Thus, if caused by microbiota changes, the result is not contradictory in that, the T factor was involved in the microbiota change, but not the presence or absence of N factor.

In this study, *Rothia* revealed a negative correlation showing a decrease with the progression of the T factor (Fig. 5C), and from a previous study, we know that it is remarkably low in OSCC patients [9,10,23]. Moreover, although this bacterium is considered to be phylogenetically related to *Actinomyces*, its pathogenicity itself is considered low. Thus, it is possible that *Rothia* is common among healthy people.

In this study, we examined factors influencing cancer incidence using logistic regression analysis (Table 3). So far, sex, smoking, and drinking habits have been reported as cancer risk factors [3,33,34], but in this study we obtained similar results only with regard to sex. An odds ratio of 10.85 also indicated a high risk.

The genera *Rothia, Alloprevotella, Capnocytophaga, Fusobacterium, Haemophilus*, and family *Peptostreptococcus*, which showed a significant difference in abundance among OSCC patients compared to the healthy controls, had no influence on cancer incidence, however the Chao 1 index, indicating α-diversity, showed a significant difference regarding cancer incidence. Although the odds ratio was 1.006, it is possible that the diversity index indicates an influencing factor for cancer incidence.

4.3. Relationship between oral microbiota and disease

It is suggested that there is also an age difference in the occurrence of oral cancer [35].

Recent studies have reported that gut microbiota and age are related and that the microbiota changes with age [36,37]. In addition, a relationship between intestinal microbiota and the general condition of the immune status has also been reported [38].

There have been several reports showing differences between the oral microbiota in healthy people and periodontitis patients [39,40]. Changes in the oral microbial flora have also been reported using saliva samples for comparison between aged care facility residents and the healthy group among the elderly in Japan. A significant difference was observed in α-diversity between these two groups [41]. From the results obtained in this study, differences in diversity and the existence of specific minor bacterial species might influence the incidence of OSCC and microbiota composition.

Although their influence is not strong, some bacteria related to dental caries and periodontal disease also suggest that the cancer risk can be reduced by maintaining good oral hygiene.

Despite the microbiota distribution evaluation at this time, it has been reported that the ratio as well as the number of bacteria influence disease onset [42]. There is a possibility of identifying new causative bacteria for the onset by comparing the number of bacteria.

In addition, bacteria as well as fungi are involved in the formation of oral microbiota. It is known that *Candida* and other fungi are detected at high proportions in the oral cavity if the immune status is reduced as in the elderly and HIV patients [43,44]. It is suggested that these fungal species are also associated with oral cancer and other cancers, but the specifics of how they are involved are not clear [45]. In recent years, analysis of microbiota using NGS has been carried out widely, but research on the characteristics of the fungal flora and their relation to the microbiota in OSCC patients will be necessary in the future.

5. Conclusion

In this study, we compared the oral microbiota of 60 Japanese oral cancer patients with 80 non-cancer individuals and found high bacterial diversity and a change in the ratio of some bacterial species.

Conflict of interest

The authors have no conflict of interest.

CRediT authorship contribution statement

Yasuharu Takahashi: Data curation, Writing - original draft. **Jonguk Park:** Formal analysis, Writing - original draft. **Koji Hosomi:** Formal analysis. **Tomonori Yamada:** Data curation. **Ayaka Kobayashi:** Data curation. **Yuji Yamaguchi:** Data curation. **Susumu Iketani:** Data curation. **Jun Kunisawa:** Formal analysis. **Kenji Mizuguchi:** Formal analysis. **Nobuko Maeda:** Writing - original draft. **Tomoko Ohshima:** Writing - original draft.

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