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RESEARCH

Bacterial distribution and inflammatory cytokines associated with oral cancer with and without jawbone invasion—a pilot study

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Abstract

Objective To explore the bacterial and inflammatory variations in oral cancer patients with and without jawbone invasion.

Materials and methods A total of 20 specimens of fresh tumor tissue, including 10 from the tumor-invaded jawbone (JIOC group) and 10 without jawbone invasion (NJIOC group), were collected from oral cancer patients. Meanwhile, 10 specimens from normal oral mucosa were collected from healthy patients (control group). The microbiomic content of each sample was analyzed by 16S rRNA gene sequencing, while the expression of inflammatory cytokines was assessed using protein microarray analysis.

Results There was a significant difference in β diversity between JIOC and NJIOC groups ($P < 0.05$), but no difference between NJIOC and control groups. The average relative abundance of *Fusobacteria* and *Spirochaetes* was higher, while *Firmicutes* was lower in the JIOC group than in the NJIOC group (all $P < 0.05$). The expression of pro-inflammatory cytokines like interleukin (IL)-1α, IL-1β, IL-4, and IL-8 was upregulated in the JIOC group compared with the NJIOC group, while MCP-1 was decreased (all $P < 0.05$). *Slackia* spp. and *Howardella* spp. were positively correlated with IL-4; *Odoribacter* spp. and *Acidaminococcaceae* spp. were negatively correlated with IL-4, and *Clostridium* XIVa spp. was negatively correlated with IL-1α and IL-1β.

Conclusion Bacterial and inflammatory differences were observed in oral cancer patients with and without jawbone invasion, where the relative abundance of the differential bacteria was associated with the expression of the inflammatory cytokines.

Clinical relevance This study investigated the changes in the flora during jawbone invasion in oral cancer and its effect on inflammatory factors, elucidating the possible mechanisms of jawbone invasion caused by oral cancer, which may lead to new ideas for the clinical prevention and treatment of jawbone invasion in oral cancer.

Keywords Oral cancer · Jawbone invasion · Microbiota · IL-1α · IL-1β · IL-4

Introduction

The oral microbiome has been found to have an important role in human health and disease, especially some particular microorganisms. These microorganisms form a complex ecological community affecting the pathogenesis and development of many oral and systemic diseases [1]. For example, the oral microbiome has been associated with oral cancer. Previous studies have suggested that oral bacteria exert their oncogenic effect in three ways [1]: causing chronic inflammation, acting as an antiapoptotic, and producing carcinogenic substances. Moreover, a correlation between specific bacteria (including *Streptococcus* sp., *Peptostreptococcus* sp., *Prevotella* sp., *Porphyromonas gingivalis*, and *Capnocytophaga gingivalis*) and oral squamous cell carcinomas (OSCCs) has been reported

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[2]. Also, studies have found that different surfaces attract distinct microbial communities because each niche provides a unique ecosystem with the optimal conditions and nutrients for its populating microbe [1, 3]. Furthermore, different stages of oral cancer seem to be associated with a different abundance of bacteria [4]. Clinically, local swelling and pain of the soft tissue and absorption of alveolar bone are often caused by bacteria-based chronic inflammation, such as periodontal diseases [5].

Oral cancer, especially gingival cancer, palate cancer, and floor of the mouth cancer neighboring the jawbone, can easily invade the jawbone. Among oral solid tumors, squamous cell carcinoma and adenoid cystic carcinoma are prone to jaw invasion. The probability of squamous cell carcinoma presenting with jaw invasion is about 12–56 % [6]. According to a study, 39.2% of patients with adenoid cystic carcinoma had positive infiltration of the tumor margins [7]. This study aimed to explore the bacterial distribution and its association with the inflammatory cytokines of OSCCs with and without jawbone invasion.

Materials and methods

Tissue collection

A total of 20 specimens of fresh tumor tissue (10 where the tumor invaded the jawbone and 10 at the front of tumor invasion but without jawbone invasion) were collected from OSCC patients (age 45–79) admitted to the Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology (PKUSHS) between December 2018 and April 2019. The inclusion criteria were as follows: (1) patients who have not systematically used antibiotics; (2) those who have not used mouthwash containing antibiotics within 1 week before specimen collection; (3) OSCC was confirmed by biopsy, and the pathological finding was evaluated by contrast-enhanced computed tomography (CT) data; (3) those who signed the informed consent that was reviewed and approved by the Ethical Committee of PKUSHS (PKUSSIRB-201,839,134). The OSCC stages were classified according to the AJCC staging manual (8th edition, 2017). First, biopsies were collected from fresh tumor tissue where the tumor invaded the jawbone or next to the front of the invasion in oral cancer patients.

In addition, the normal mucosa tissue from 10 patients (ages 25–78) who underwent other oral surgery, including curettage of jaw cysts and extraction of impacted teeth (without any infection), was used as normal control.

Fresh tissue was then placed in a sterile vial on ice and snap-frozen in liquid nitrogen until further use.

DNA extraction

QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) was used to extract DNA from each tissue sample. Briefly, 200 mg of the tissue was mixed with 1 mL of InhibitEX Buffer and a proper amount of glass beads (0.5 mm diameter, Qiagen). Then, the mixture was shaken for 1 min twice with a homogeneous instrument (FASTPREP-24, Aosheng Biotech, China). Afterward, the DNA purification was performed according to the manufacturer's instructions.

16S rRNA gene sequencing

The V3-V4 region of the bacteria 16S ribosomal RNA genes was amplified using barcoded primer 806R 5′-GGACTACVSGGGTATCTAAT-3′ and 341F 5′-CCTACGGGRSGCAGCAG-3′ (6). The following PCR conditions were applied: 95 °C 3 min, followed by 30 cycles at 98 °C for 20 s, 58 °C for 15 s, and 72 °C for 20 s, and extension at 72 °C for 5 min. PCR reactions were performed in a 30-μL mixture containing 1 μL of each primer (10 μM), 15 μL of 2× KAPA Library Amplification ReadyMix, 50 ng of template DNA, and ddH_2O. In addition, negative controls (consisting of empty sterile storage tubes) were processed for DNA extraction. Amplification was performed using the same procedures, and reagents were used for the mucosa samples. There was no detectable amplification in the negative controls.

Amplicons were extracted from 2% agarose gels. AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) was used for sample purification, and Qubit®2.0 (Invitrogen, USA) was used for quantification. All quantified amplicons were pooled to equalize concentrations for sequencing using Illumina MiSeq/PE250 (Illumina, Inc., CA, USA). The paired-end reads of 425 bp were overlapped on their 3 ends for concatenation into original longer tags using PANDAseq (https://github.com/neufeld/pandaseq, version 2.9). Sequencing, DNA extraction, and library construction and sequencing were conducted at Realbio Genomics Institute (Shanghai, China).

Process of sequencing data

Sequencing data were processed as previously described [8]. Briefly, assembled tags, trimmed of barcodes and primers, were further checked on their rest lengths and average base quality. 16S tags were restricted between 220 bp and 500 bp such that the average Phred score of bases was no worse than 20 (Q20) and no more than three ambiguous N. The copy number of tags was enumerated, and redundant tags were removed. Only the tags with a frequency > 1, which tended to be more reliable, were clustered into

OTUs, each of which had a representative tag. Operational taxonomic units (OTUs) were clustered with 97% similarity using UPARSE (http://drive5.com/uparse/). Chimeric sequences were identified and removed using Usearch (version 7.0.1090). Each representative tag was assigned to taxa by the RDP Classifier (http://rdp.cme.msu.edu/) against the RDP database (http://rdp.cme.msu.edu/) using a confidence threshold of 0.8. OTU profiling table, and alpha diversity analyses were also achieved by Python scripts of QIIME 2 (version 2022.8).

Protein microarray analysis

Protein microarray analysis was performed as previously described [9]. Briefly, human protein antibody arrays (Quantibody® Human Inflammation Array 1, Cat. #QAH-INF-1, RayBiotech, Inc., Guangzhou, China) were provided by RayBiotech. There were positive control spots and negative control spots on the array. The positive control spots were standardized amounts of biotinylated IgGs directly printed onto the array. The signal intensity data were calculated as the fluorescence intensity minus the local background and normalized to the positive control signals. In addition, the significance of the differences in the expression of 10 cytokines between JIOC samples and NJIOC samples was evaluated via protein microarray analysis. The expression ratio represents the tendency of a difference in protein expression.

Statistical analysis

GraphPad Prism 8 with one-way ANOVA and Wilcoxon tests was used for statistical analysis. Spearman's correlation was used to examine correlations between cytokines and species at the genus level using the corrplot package in R. A P value < 0.05 was considered statistically significant.

Results

This study enrolled 30 patients grouped into JIOC (jawbone invasion of oral cancer), NJIOC (non-jawbone invasion of oral cancer), and control (healthy controls) groups (10 patients per group). Of the 20 patients with OSCC, 10 NJIOC cases without jawbone invasion were in the early stage (T1,2), and the tumor was mainly located in the tongue (three cases) or buccal mucosa (seven cases). On the other hand, 10 patients in the JIOC group with jawbone invasion were in the advanced stage (T3,4), and the tumor mainly occurred in the gingiva (eight cases) and the floor of the mouth (two cases). The occurrence of lymph node metastasis in the two groups was the same, and two patients in each group developed lymph node metastasis. The patients' information is shown in Table 1.

16S rRNA gene amplicons were sequenced from the 30 qualified samples. After processing, 393,923 sequences were generated (mean, 13,131 ± 2,897; range, 6,594 to 18,491). Using the minimum (6594) and a 97% similarity level, OTUs were sub-sampled and detected. In addition, the community richness (Chao1 and observed species) and community evenness (Shannon index and Simpson index) were detected to describe and compare the alpha diversity between these groups. There were no significant differences in these four indexes (Fig. 1a–d).

Beta diversity was measured to display the relative microbiome spaces between the three groups through principal coordinate analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA). PCoA results showed that the JIOC group, the NJIOC group, and the control group could be separated based on weighted unifrac and unweighted unifrac (Fig. 1e, f). In addition, PERMANOVA analysis results showed significant differences between the JIOC group and the NJIOC group patients ($P < 0.001$) but no difference between the NJIOC group and control group patients ($P > 0.05$) (Fig. 1g–i).

The relative abundance of microbial taxa characterized the microbial community composition. Sixteen phyla, 30 classes, 58 orders, 111 families, and 211 genera were detected in all specimens, and the main phyla were Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, Spirochaetes, and Actinobacteria (Fig. 2). There was no significant difference in species composition between the JIOC and the NJIOC groups at the phylum and order level. Yet, compared with the NJIOC group, the average relative abundance of *Firmicutes* was lower in the JIOC group, while *Fusobacteria* and *Spirochaete*s were higher (all $P < 0.05$). At the order level, the average relative abundance of *Bacteriodales*, *Fusobacteriales*, *Spirochaetales*, *Selenomonadales*, *Campylobacterales*, and *Lactobacillaveles* was higher in the JIOC group, and *Clostridiales*, *Flavobacteriales*, and *Neisseriales* were lower ($P < 0.05$, Fig. 3).

According to the hierarchical relationship, we drew the phylogenetic trees to reflect the genetic relationship between the JIOC and NJIOC groups. The LEfSe (linear discriminant analysis effect size) analysis showed 14 kinds of bacteria that were different between the two groups (LDA score > 3.0, $P < 0.05$; Fig. 4).

The Quantibody® Human Inflammation Array 1 was used to show the differences in ten cytokines between the two groups. The JIOC group had a higher expression level of IL-1α, IL-1β, IL-4, and IL-8 and a lower expression level of MCP-1 compared with the NJIOC group. Moreover, the other cytokines did not differ between the two groups, including IFN-γ, TNF-α, IL-6, IL-10, and IL-13 (Fig. 5).

Table 1 Clinical characteristics of the patients in this study

Characteristics	Healthy control	OSCC	
		Without jawbone invasion (NJIOC)	With jawbone invasion (JIOC)
Number of patients	10	10	10
Age (years)			
Range	29–80	49–70	45–79
Mean ± SD	53.9 ± 17.0	57.8 ± 6.9	63.5 ± 10.3
Gender			
Male	4	9	6
Female	6	1	4
T stage			
T1,2	-	10	0
T3,4	-	0	10
N stage			
N (−)	-	8	8
N (+)	-	2	2
Overall stage			
II	-	8	-
III	-	2	-
IV	-		10
Alcohol drinking			
No	8	7	7
Yes	2	3	3
Cigarette smoking			
No	7	4	6
Yes* (median pack-years)	3 (1.5)	6 (20)	4 (25.5)
Site			
Gingiva	7	-	8
Mouth floor	-	-	2
Tongue	1	3	-
Palate	1	-	-
Buccal mucosa	1	7	-

*The median pack-years of smoking among subjects in the healthy control group, NJIOC group, and JIOC group were 1.5, 20, and 25.5, respectively

The correlation analysis showed that IL-1α and IL-1β were negatively correlated with *Clostridium* XIVa spp. IL-4 was positively correlated with *Slackia* spp. and *Howardella* spp. and was negatively correlated with *Odoribacter* spp. and *Acidaminococcaceae* spp.; MCP-1 was positively correlated with *Odoribacter* spp. and negatively correlated with *Slackia* spp. (Fig. 6).

Discussion

According to the World Health Organization (WHO), there were 377,713 new cases of cancers of the oral cavity and lips and 177,757 deaths in 2020 [3]. OSCCs constitute > 90% of oral and oropharyngeal cancer, and its main etiological factor is the synergistic effect of tobacco and alcohol use [10]. Yet, the possible causal relationship between microbes-inflammation-cancer has also been suggested. Several studies have found that the accumulation of microorganisms promotes the release of compounds that produce inflammation in the gingival tissue [11, 12]. Moreover, Socransky *et al.* discovered that colonization of certain anaerobic subgingival bacteria promotes both the onset and the development of periodontitis [13], correlated with an increased overall risk of oral cancer [14]. Also, the radiological parameter "mean bone loss" was identified as an independent risk factor for OSCC. A previous study found that periodontal treatment significantly reduces the risk of OSCC presence [15].

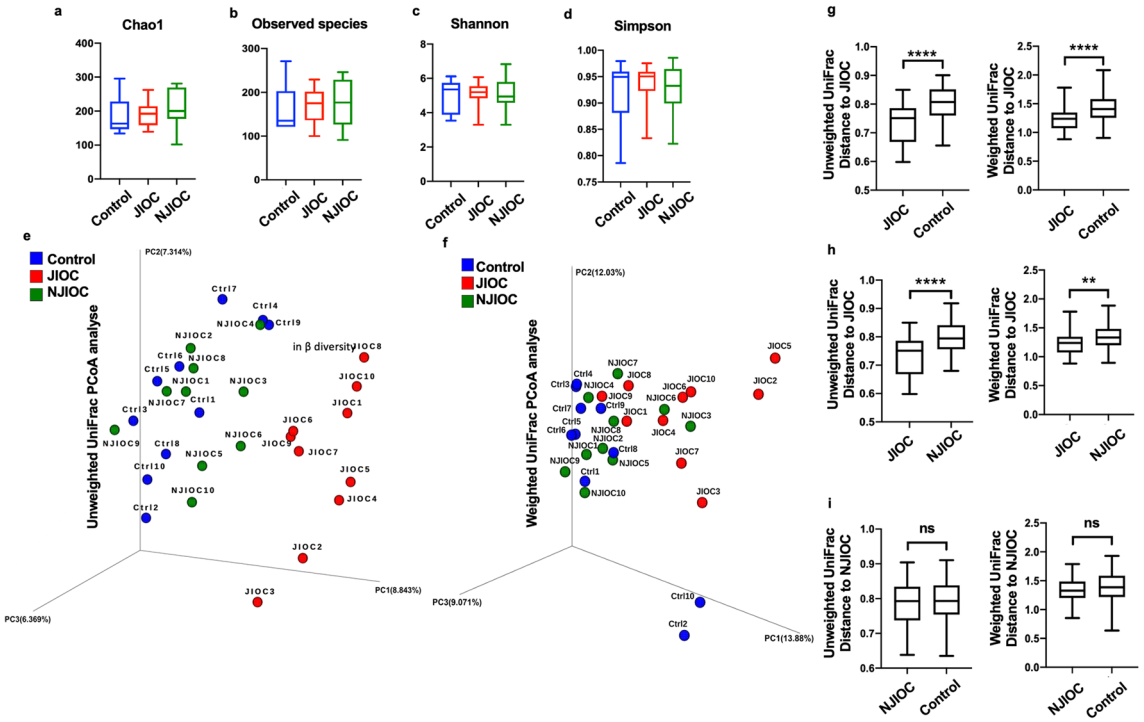


Fig. 1 Analysis of species diversity in the JIOC, NJIOC, and control groups. **a**–**d** The α-diversity presented as Chao1, observed species, Shannon, and Simpson indexes. **e** and **f** represented the unweighted unifrac and weighted unifrac, respectively. The red, green, and blue circles display the sample positions of the JIOC group, the NJIOC group, and the control group, respectively. The figure showed the distinct zones between the same samples and others. **g** The difference between JIOC and control in β diversity had statistical significance by PERMANOVA. **h** reflected the differences between JIOC group and NJIOC group in β diversity. **i** The β diversity had no statistical significance between NJIOC group and control group. (**$P < 0.01$; ****$P < 0.0001$; ns, no significant)

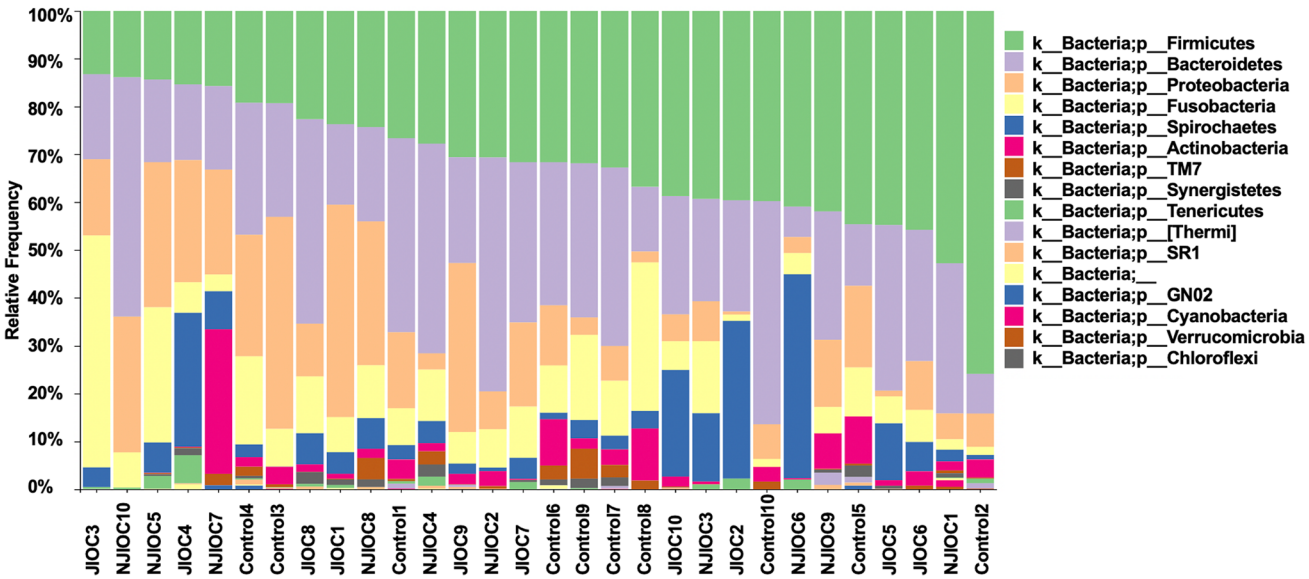
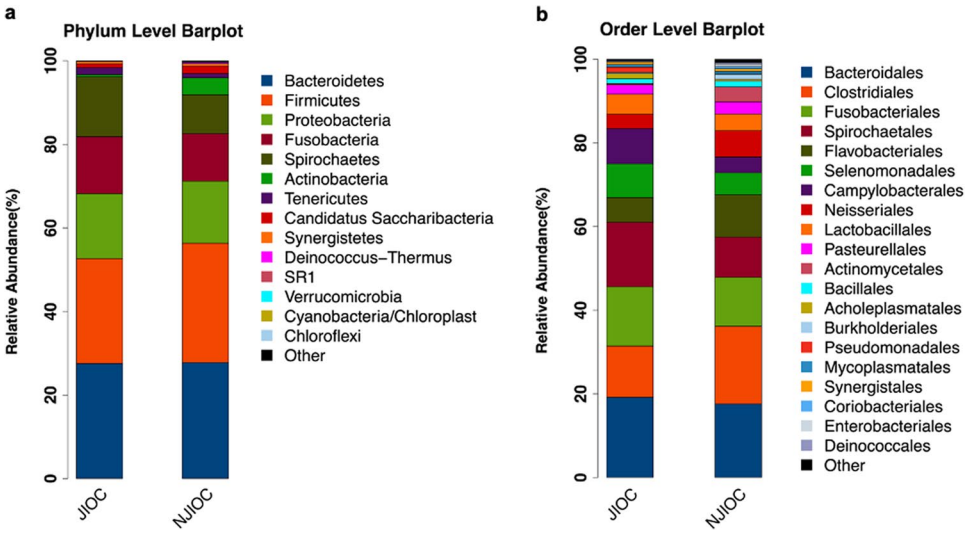


Fig. 2 Species stacked bar chart of 30 samples in phylum level. The outline of the species and abundance composition of species classification subjects were described



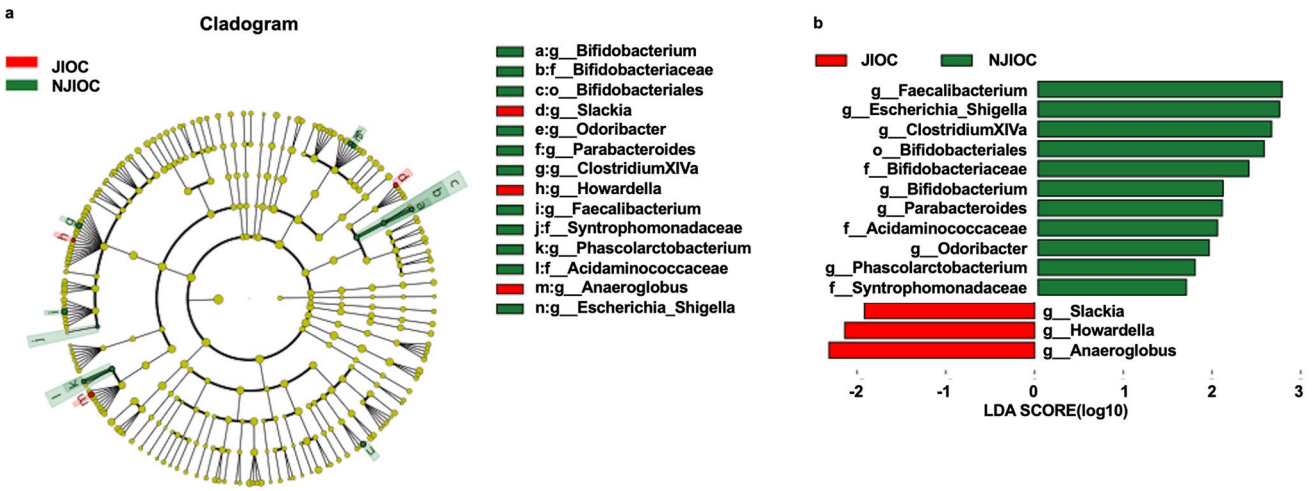


Fig. 3 Relative abundance of different bacteria in phylum and order level

Fig. 4 Significant differential species between JIOC group and NJIOC group. **a** The cladogram showed that 14 kinds of genera were significantly different between these two groups. **b** showed the LDA scores of the differentially abundant taxa

The microbial community in tumor tissue is different from that in normal tissue. With the progress of the tumor, the microbial community in OSCC also changes [4]. In their review, Irfan *et al.* also states that many studies have shown that microbiomes may promote carcinogenesis through chronic inflammation [16]. The increase of IL-1β in saliva further confirmed the association between inflammation induced by bacteria and the development of oral cancer [17]. IL-1β causes osteoclast formation and bone resorption, which leads to local inflammatory changes in the periodontium [2, 18]. However, the mechanisms through which periodontitis and oral microbiome affect OSCC progression are still not fully understood. Therefore, obtaining bacterial information and the corresponding inflammatory state in the OSCC with jawbone invasion may have an important value for diagnosing and treating OSCC patients.

According to the AJCC TNM classification of malignant tumors, tumors invading bone are upstaged to T4 independently of size, location, or type of bone invasion [19]. A previous study found that a relative amount of *Anaeroglobus geminatus* spp. increases in people suffering from periodontitis and apical periodontitis [20]. Moreover, a recent study reported that *Howardella* spp. could suggest caries-active and caries-free supragingival plaques in permanent dentition

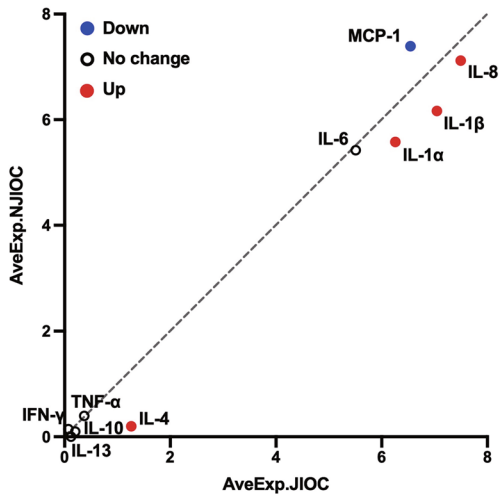


Fig. 5 Cytokine secretion levels from JIOC and NJIOC. Scatter plot generated from protein micro-array data reflecting differential expression proteins (DEPs) between the JIOC and NJIOC. The basic statistics used for significance analysis are Wilcoxon tests. Red presents upregulation, blue presents downregulation, and black shows no difference

[21]. Johnson *et al.* found that *Slackia* spp. is significantly increased in OSCC compared with epithelial precursor [22].

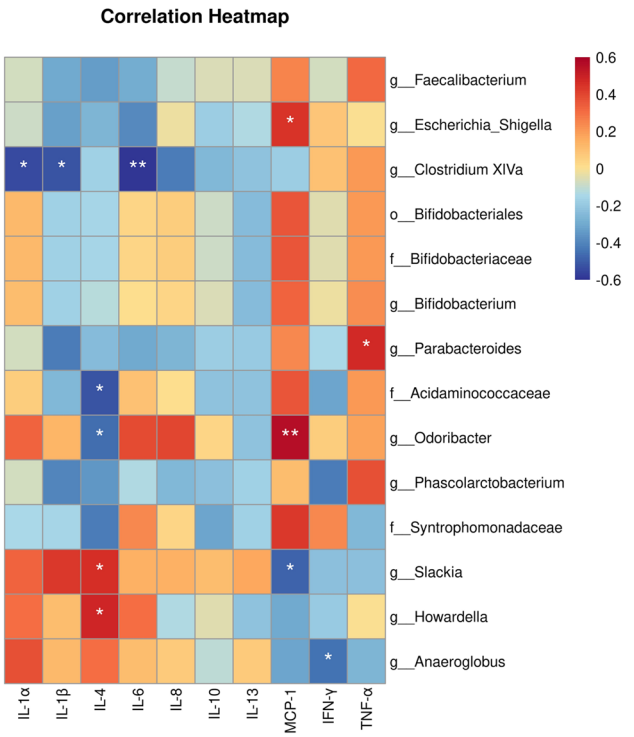


Fig. 6 Heat map reflecting the correlation between inflammatory cytokines and differential species at the genus level. Colors reflect the correlation coefficient from positive correlation (red) to negative correlation (blue). (*$P < 0.05$; **$P < 0.01$)

In this study, we found that *Anaeroglobus* spp., *Howardella* spp., and *Slackia* spp. had higher relative abundance in the JIOC group compared with the NJIOC group, suggesting that these three species may promote the occurrence of jawbone invasion in OSCC (Fig. 4b).

Some *Parabacteroides* spp. species can reduce the release of IL-6, IL-8, IL17, and gamma interferon to reduce inflammation [23, 24]. *Bifidobacterium* spp. can activate DC to secrete IL-10 and lead to the low production of pro-inflammatory cytokines IL-12 [25, 26]. *Faecalibacterium prausnitzii* spp. positively affects the body and produces anti-inflammatory factors [27]. Lima *et al.* found that *Odoribacter splanchnicus* spp. protected immune cells from colitis [28], which is consistent with the results observed in this study.

Hashim and colleagues found that oral hygiene has an active role in reducing the risk of head and neck cancer [29]. Cytokines have a positive effect on the occurrence of chronic inflammation through pro-inflammatory cytokines [30]. Inhibition of the signaling pathway of both NF-kB and MAPK and regulating the expression of IL-8 are ways for *Shigella* spp. to inhibit inflammation [31]. *Parabacteroides distasonis* spp. exert anti-inflammatory properties by relieving IL-8 release and is related to the level of IL-1β [32, 33]. Many studies have shown that periodontitis could be induced by pro-inflammatory cytokines, such as IL-1α, IL-1β, and TNF-α [34–36]. These cytokines influence osteoclasts' formation and absorption capacity in different ways, leading to bone resorption [37, 38]. Goertzen et al. showed that the pro-inflammatory cytokines IL-1α, IL-1β, IL-6, IL-8, and TNF-α were significantly increased in saliva samples from patients with oral cancer [39]. TNF-α has been shown to stimulate the invasion of oral squamous cell carcinoma. However, in this study, IL-6 and TNF-α were not significantly increased in oral cancer jaw invasion samples, which may be due to differences in sample types or because we used a small sample size that did not fully characterize the expression of inflammatory cytokines. IL-4 may be involved in osteoclastogenesis actively by inducing $CD4^+$ T cells to differentiate into Th2 cells to secrete osteoclastogenic cytokines [40, 41]. In this study, we found that *Clostridium* XIVa spp. was negatively correlated with both IL-1α and IL-1β. Our results also showed that *Slackia* spp. was positively correlated with IL-4 and negatively correlated with MCP-1 (Fig. 6).

Conclusions

The relative abundance of specific bacteria in tissues was altered when jawbone invasion occurred in oral cancer, and this alteration was strongly correlated with the progression of inflammation, thus suggesting that specific bacteria may

promote jawbone invasion in oral cancer through inflammation responses.

Study limitations

The present study assessed the bacterial flora characteristics associated with a jaw invasion of oral tumors. Yet, this study was a single-center study with a small sample size, which may lead to certain data bias. To judge the impact of sample size on the study conclusions, we used online software (http://powerandsamplesize.com) to confirm the weak impact on the study results while calculating β diversity. Still, this data has given some important clues on the direction to take. Thus, we are currently designing the next study with a larger sample size, including more experimental verification, to confirm these findings further.

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Author contributions Yuxing Guo, Yuanning Yang, and Qingxiang Li contributed to the conception, design, and data acquisition; drafted the manuscript; and critically revised the manuscript. Qiao Qiao, Ning Zhao, Hongyuan Huang, and Ying Zhou contributed to the design, data acquisition, drafting the manuscript, and critical revision of the manuscript. Chuanbin Guo contributed to the design, interpretation, drafting of the manuscript, and critical revision of the manuscript. All authors gave final approval and agreed to be accountable for all aspects of the work.

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Declarations

Ethics approval Approval was obtained from the ethics committee of the Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology (PKUSHS). The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Consent to participate Informed consent was obtained from all individual participants included in the study.

Consent for publication The participants have consented to the submission of the case report to the journal and signed informed consent regarding publishing their data.

Conflict of interest The authors declare no competing interests.

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