**Fusobacterium is enriched in oral cancer and promotes induction of programmed death-ligand 1 (PD-L1)**

Chieko Michikawa a; Vancheswaran Gopalakrishnan b; Amani M. Harrandah c; Tatiana V Karpinet c; Rekha Rani Garg d; Randy A. Chu e; Yuk Pheel Park b; Sasanka S. Chukkapallia f; Nikhita Yadlapalli f; Kelly C. Erikson-Carter g; Frederico Omar Gleber-Netto h; Elias Sayour i; Ann Progulske-Fox j; Edward K. L. Chan k; Xiaogang Wu l; Jianhua Zhang m; Christian Jobin n; Jennifer A. Wargo o; Curtis R. Pickering p; Jeffrey N. Myers q; Natalie Silver r.

*Department of Head and Neck Surgery, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA
1 Department of Maxillofacial Surgery, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan
2 Department of Surgical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA
3 Department of Oral Biology, University of Florida College of Dentistry, Gainesville, Florida, USA
4 Department of Oral Biology, Univ AlQura University, Makkah, Saudi Arabia
5 Department of Genomic Medicine, The University of Texas MD Anderson Cancer Center, Houston, Texas, USA
6 Department of Pediatrics, University of Florida, Gainesville, Florida, USA
7 Institute for Personalized Cancer Therapy, The University of Texas MD Anderson Cancer Center, Houston, TX, USA
8 Department of Pathology, Immunology and Laboratory Medicine, University of Florida, Gainesville, Florida, USA
9 Department of Neuroscience, Baylor College of Medicine, Houston, TX, USA
10 Department of Pediatrics and Neurosurgery, University of Florida, Gainesville, Florida, USA
11 Department of Internal Medicine, University of Florida, Gainesville, FL, USA
12 Cleveland Clinic, Head and Neck Institute/Lerner Research Institute, Cleveland, OH, USA

**Abstract**

Recently, increased number of studies have demonstrated a relationship between the oral microbiome and development of head and neck cancer, however, there are few studies to investigate the role of oral bacteria in the context of the tumor microenvironment in a single head and neck subtype. Here, paired tumor and adjacent normal tissue samples from thirty-seven oral tongue squamous cell carcinoma (SCC) patients were subjected to 16S rRNA gene sequencing and whole exome sequencing (WES), in addition to RNA sequencing for tumor samples. We observed that *Fusobacterium* was significantly enriched in oral tongue cancer and that *Rothia* and *Streptococcus* were enriched in adjacent normal tissues. A decrease in alpha diversity was found in tumor when compared to adjacent normal tissues.

**Abbreviations:** PD-L1, programmed death ligand 1; SCC, squamous cell carcinoma; WES, Whole Exome sequencing, MDACC, The University of Texas MD Anderson Cancer Center; OUT, operational taxonomic unit; DFS, disease-free survival; OS, overall survival; FBS, fetal bovine serum, MEM, Minimum Essential Medium; ssp., subspecies; F., Fusobacterium; T. denticola, Treponema denticola; T. forsythia, Tannerella forsythia; MOI, multiplicity of infection; qRT-PCR, real-time quantitative polymerase chain reaction; LEfSe, linear discriminant analysis of effect size; TMB, tumor mutation burden; IL, interleukin; PD-1, programmed cell death protein 1.

* Corresponding author at: Cleveland Clinic, Head and Neck Institute/Lerner Research Institute, 9500 Euclid Ave/A71, Cleveland OH 44195.

E-mail address: SILVERN@ccf.org (N. Silver).

Received 1 February 2022; received in revised form 20 May 2022; accepted 1 June 2022

© 2022 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)
https://doi.org/10.1016/j.neo.2022.100813
While increased *Fusobacterium* in tumor samples was not associated with changes in immune cell infiltration, it was associated with increased PD-L1 mRNA expression. Therefore, we examined the effects of *Fusobacterium* on PD-L1 expression in head and neck SCC cell lines. We demonstrated that infection with *Fusobacterium* species can increase both PD-L1 mRNA and surface PD-L1 protein expression on head and neck cancer cell lines. The correlation between *Fusobacterium* and PD-L1 expression in oral tongue SCC, in conjunction with the ability of the bacterium to induce PD-L1 expression in vitro suggests a potential role for *Fusobacterium* on modulation of the tumor immune microenvironment in head and neck cancer.

*Neoplasia* (2022) 31, 100813

**Keywords:** Head and neck cancer, Oral cancer, Periodontal bacteria, *Fusobacterium*, PD-L1, Microbiome

**Background**

Oral cavity squamous cell carcinoma (SCC) often presents at an advanced stage and has a poor prognosis of less than 50% at 5 years [1]. Smoking and alcohol use are the predominant risk factors of oral cavity cancer in the United States [2]. In South Asia and the Pacific region, tobacco chewing with or without areca (betel) nut has also been found to be a major risk factor [3]. Nevertheless, there are increased numbers of these cancers arising in individuals without significant exposure to traditional risk factors, leaving questions about the pathogenesis of these tumors unanswered [4-6]. Furthermore, genomic analyses of oral tongue cancers from young non-smoking patients appear similar to those of older smoking patients [5]. This may indicate that the functional impact of smoking on oral carcinogens is not entirely understood and suggests that other factors including periodontal disease, [7] and the oral microbiome, [8-10] may play important roles in the development of oral cavity SCC.

The oral cavity offers several different sites as microbial niches and has a diverse microbiome with over 700 known species of bacteria, which maintains a dynamic relationship with the human host [11]. When there are imbalances in the diversity and relative proportions of species or taxa in the oral cavity (e.g. from lifestyle changes such as smoking, diet, or poor oral hygiene), inflammatory responses along with disruption of the host immune homeostasis can occur (dysbiosis); exemplified by periodontal disease [11,12]. A recent meta-analysis demonstrated that periodontal disease is an independent risk factor for oral cancer after adjustment of confounders such as tobacco, alcohol, age, and gender [7].

*Fusobacterium* has been not only associated with development of colorectal carcinoma [13], but also identified as one of the significantly enriched periodontal pathogens in oral SCC compared to control samples [8,9]. Additionally, *Fusobacterium* has been shown to create a pro-inflammatory microenvironment and shape the immune microenvironment to allow tumor propagation [13,14].

In this study, we investigated the bacterial taxonomy/abundances and assessed the association with the host genomic and immune microenvironment in oral tongue cancer using 16S rRNA gene sequencing, Whole Exome sequencing (WES), and RNA sequencing. Based on our findings, we examined the effect of *Fusobacterium* infection on expression of PD-L1 (CD274/B7-H1), in human head and neck SCC cell lines in vitro.

**Materials and Methods**

**Patient cohort and sample collection**

Thirty-seven patients from The University of Texas MD Anderson Cancer Center (MDACC) were identified for examination of tumors and paired adjacent normal tissues which were resected in the operating room, classified via pathology review, flash frozen, and stored at –80°C. The study was conducted under an approved MDACC Institutional Review Board (IRB) protocol. Adjacent normal tissues were collected from the surgical margin. Patients with a diagnosis of oral tongue SCC and receiving primary surgery with curative intent at MDACC between January 2010 and June 2015 were included. Clinico-pathological information; demographics, tumor characteristics, lymph node status, disease recurrence and follow-up status were collected for all patients (Additional file 2: Tables S1).

**16S rRNA gene sequencing**

From seventy-four paired tumor and paired adjacent normal samples, genomic DNA was isolated, and a library was prepared to perform 16S rRNA gene sequencing on MiSeq (Illumina Inc., San Diego, CA) through the collaboration with Evelo Biosciences. The sequencing raw reads were re-analyzed using an internally established bioinformatic pipeline [15,16] to generate and classify operational taxonomic units (OTUs), and to compute alpha and beta diversity metrics. Details are described in Additional file 1.

**WES and RNA sequencing**

WES was carried out on a HiSeq 2000 (Illumina Inc., San Diego, CA) for 36 tumor samples and paired adjacent normal samples (as control) from same patients. We also isolated total RNA from same 37 patient tumor samples and prepared libraries to perform RNA sequencing on a HiSeq 2500 (Illumina Inc., San Diego, CA) to characterize the tumor immune microenvironment. We did not conduct RNA sequencing for normal tissues. Details are described in Additional file 1.

**Statistical analysis for human samples**

Precise information of the bioinformatic analyses is described in Additional file 1. The Mann-Whitney (MW) U test or the spearman’s rank correlation test was used for comparisons between binary outcome variables. The Fisher’s exact test was used when proportions were compared between binary variables. Disease-free survival (DFS) and overall survival (OS) was estimated using the Kaplan-Meier method. DFS time was defined as the interval between the date of the surgery and the date of the development of local, regional recurrence or distant metastasis. OS time was calculated from the date of the surgery to the date of death, last contact, or the 5-year follow-up. All differences in groups were considered statistically significant if p<0.05. Analyses were conducted in JMP®12, SAS Institute Inc., Cary, NC, USA and R.
Cell culture

K562 (a human erythroleukemic cell line; ATCC, CCL-243TM) were grown in Iscove’s Modified Dulbecco’s Medium, supplemented with 10% fetal bovine serum (FBS). The following six human head and neck SCC cell lines were used; HN, CAL27, BHY, FaDu, OQ01, and RPMI2650. HN and BHY cell lines were purchased from DSMZ (Braunschweig, Germany) and grown in 90% Dulbecco’s Minimum Essential Medium (MEM, 4.5g/L glucose) and 10% heat inactivated FBS. CAL27 (ATCC, CRL-2095) was grown in Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS. FaDu (ATCC, HTB-43TM) was grown in Eagle’s MEM supplemented with 10% FBS. OQ01, a primary cultured oral tongue SCC cell line was provided by Dr. Lung-Ji Chang, University of Florida [17]. OQ01 cells were cultured in DMEM/F12 supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 units/ml penicillin. RPMI 2650 (ATCC, CCL-30) were grown in MEM supplemented with 1.5 g/liter sodium bicarbonate, 1.0 mM sodium pyruvate, and 0.1 mM non-essential amino acids. All cell culture was under standard tissue culture conditions (37°C, 5% CO2).

Growth conditions for bacterial strains

Periodontal bacteria; *Fusobacterium (E.) nucleatum* ssp. *vincentii* (E. vincentii), (ATCC, 49256), *F. periodonticum* (ATCC, 33693), *T. denticola* (T. denticola), (ATCC, 35404) and *T. forsythia* (T. forsythia), (ATCC, 43037) were grown as previously described under anaerobic conditions at 37°C in an anaerobic chamber in antibiotic free medium [18]. Bacteria were grown in culture medium until the logarithmic growth phase with growth rate measured at optical density (OD550nm). Culture characteristics and gram stains were assessed for all cultures prepared in this study.

Bacterial Infection of cell lines

Cancer cells were seeded at 5 × 10^3/mL in 6 well plates at 37°C overnight in antibiotic free medium. Bacteria were added at a multiplicity of infection (MOI) of 100. For controls, cell lines were cultured in medium alone. Plates were incubated at 37°C for 24 h as previously described [19]. Cell lysates were collected and stored frozen at -80°C for RNA isolation or prepared fresh for flow cytometry.

Quantification of mRNA expression level by real-time quantitative polymerase chain reaction (qRT-PCR)

Total RNA from harvested cells was isolated using the mirVana isolation kit (Ambion, Austin, TX) following the manufacturer’s protocol. RNA was concentrations were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technology Inc., Wilmington, DE). For mRNA analysis, a High Capacity cDNA RT Kit (Applied Biosystems) and TaqMan gene expression assays for target genes were used. The relative levels of RNA expression in treated samples in comparison to untreated controls (with normalization to housekeeping gene 18S or beta actin) were quantified using the comparative CT (2^-ΔΔCT) method. Data collected from three independent biological replicates are reported as average log2-foldchange of replicates +/- SEM. Differences in treated and untreated samples were analyzed for statistical significance using Student’s t-test with * p ≤ 0.05, ** p ≤ 0.01, and *** p ≤0.001.

Flow cytometry

Adherent cells were washed with PBS and incubated in 0.05% Trypsin-EDTA for 5-10 min in a 37°C incubator to detach cells. After centrifugation, the cells were collected and counted. Cells were stained for live and dead for 20-25 min followed by one wash with PBS and centrifugation. Later, the cell pellet was re-suspended in 100ul of FACS buffer, and stained with PE-labeled anti-PD-L1 antibody for 1h on ice and then cells were washed twice with PBS. Cells were fix in 2% paraformaldehyde, and were acquired on FACSCanto II instrument (BD, Heidelberg, Germany) Flow cytometry data was analyzed using FlowJo software (FlowJo, Ashland, OR).

Statistical analysis for in vitro data

All cell line experiments were done in triplicate, and two tailed t-test with Welch’s correction (unpaired) was used to compare groups using GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA). Error bars are representative of mean ± SEM between experimental replicates. All differences in groups were considered statistically significant if p<0.05.

Results

Tumors have decreased species diversity and increased Fusobacterium relative to adjacent normal

Among seventy-four paired tumor and normal samples sequenced, five samples failed sequencing and were excluded leaving 69 samples (33 tumor and 36 normal samples) useful for analysis (Additional file 2: Tables S2-3 and Additional file 3: Figure S1). Of note, the unpaired data had no strong selection bias in relative abundances for downstream analysis. We therefore used all data for taxonomic composition profiling, assessing diversity, and exploring differentially abundant taxa, but used data from 33 tumor samples for correlation with clinical outcomes, genomic, and RNA information.

We first determined the taxonomic profile of bacteria in both tumor and adjacent normal tissues. The top 5, 10, and 15 bacterial taxa at the phylum, genus, and species level, respectively, were selected by combined value of relative abundances in 69 samples (Fig. 1A-E and Additional file 3: Figures S2A-D). In all levels, there were large inter-individual variations for both tumor and adjacent normal specimens in terms of relative abundances, which is consistent with prior observations of healthy human samples [20]. We observed 492 species in our samples including F. periodonticum and four subspecies of F. nucleatum; ssp. vincentii, ssp. polymorphum, ssp. nucleatum, and ssp. animalis. These subspecies were combined into F. nucleatum for downstream analysis.

Normal tissue had higher alpha-diversity than tumor tissues using the Chao1 index (p = 0.023) which considers number of species, but the difference was modest by other indices that consider richness and evenness of species (Fig. 2A). Beta-diversity by principal coordinate analysis using weighted UniFrac distances [21] and the Bray-Curtis dissimilarity [22] showed that tumor and normal tissues had significantly different overall taxonomic composition (Fig. 2B). Association network of samples calculated by Anets algorithm [23] revealed that tumor and normal samples of the same patient had very similar profiles of the shared richness across the samples (Fig. 2C), suggesting that tumor and normal tissues share a majority of OTUs. These results are consistent with the taxonomic profile that inter-individual variation of taxonomic proportions has marked differences, even at species level, but that major taxonomic types overlapped between tumor and normal (Fig. 1A-E and Additional file 3: Figures S2A-D). We then assessed differentially abundant bacteria using linear discriminant analysis of effect size (LEfSe) [24], (Fig. 2D). LEfSe method revealed that genus *Fusobacterium* was enriched in tumor tissue up to phylum level. *F. nucleatum* was not calculated as differentially enriched taxa but had the largest relative abundance in tumor and third relative abundance in normal. Species of *Rothia* and *Streptococcus* were enriched in normal tissue up to phylum and class level, respectively.
Next, we assessed if differentially abundant taxa affect the alpha-diversity of tumor samples using relative abundance of *Fusobacterium*, *Rothia*, and *Streptococcus* in tumor tissues and also those strains, if they were calculated by the LEfSe method and found in greater than 60% of prevalence among 33 tumor cases. Relative abundances were classified into two groups; low or high based on the median value hereafter analysis.

There was no significant correlation between alpha-diversity and *Fusobacterium* using Chao1 and Shannon indices, but there was a significant reverse correlation between *Fusobacterium* and alpha diversity using the Simpson index (p = 0.05, Fig. 2E). *Rothia* and *Streptococcus* at the species level showed significant correlation with alpha-diversity in all indices (Additional file 3: Figures S3A-C).

We then investigated whether *Fusobacterium* was associated with clinico-pathological variables. We observed no significant correlation between gender, age, smoking status, alcohol use, tumor size, or pathological nodal status and relative abundance of *Fusobacterium*, *Rothia*, or *Streptococcus* in tumor (Additional file 2: Table S4 and Additional file 3: Figure S4).

**TP53 mutational status correlates with Rothia abundance and TMB**

Using WES data, we characterized somatic mutational status for 32 out of 36 tumors which have taxonomic data, and generated tumor mutation burden (TMB) (Additional file 2: Tables S5-6). TP53 mutations were found in 18 samples (56%), CDKN2A mutations were in 6 samples (19%), FAT1 and CASP8 mutations were observed in only 2 samples (6%), while NOTCH1 and PIK3CA mutation was found in 1 sample (3%). The median value of TMB was 0.74 mutations/megabase with a range of 0.05 to 3.37. Of note, TMB was independent from smoking habit in our cohort (Smoking habit; Never versus Ever, p = 0.36).

We focused our attention on TP53 mutational status due to the small proportion of other gene mutations and investigated association with our microbiota analysis. We observed no significant differences in relative abundances of *Fusobacterium* and *Streptococcus* in tumor according to TP53 mutation status (Additional file 2: Table S7). However, tumors with TP53 mutations had significantly lower relative abundances of *Rothia* (p = 0.004, Additional file 2: Table S7) and lower tumor alpha-diversity (Fig. 3) compared to tumor with TP53 wild-type. Additionally, tumor alpha-diversity showed significantly reverse association with TMB (Additional file 2: Table S8).

**Fusobacterium abundance correlates with CD274 (PD-L1) expression but not immune cell infiltration in tumor**

We next explored if *Fusobacterium* may play a role in modulation of the tumor immune microenvironment for 33 out of 37 tumors which have OTUs (Additional file 2: Table S9). We found significant association between increased PD-L1 expression status and increased *Fusobacterium* abundance (p = 0.03, Fig. 4). Since PD-L1 was the only candidate biomarker investigated in this study, we did not perform an FDR correction on the p-value. However, we did not observe any associations between *Fusobacterium* relative abundances and a variety of immune cells, including T cells (overall), CD8 T cells, Cytotoxic lymphocytes, B cell lineage, NK cells, Monocytic cell lineage, Myeloid dendritic cells, and Neutrophils (Additional file 2: Table S10-11, and Additional file 3: Figure S5). We also found no association between *Fusobacterium* abundance and expression of pro-inflammatory cytokines/signaling molecules [ interleukin 6 (IL-6), IL-18, IL-8, NF-κB, COX-2, and MMP3] which have been reported as associated with presence of *Fusobacterium* in other reports (Additional file 2: Table S12) [13,14]. Rothia or *Streptococcus* showed no significant correlations with PD-L1, all immune cells, or the pro-inflammatory cytokines/signaling except a significant reverse relationship between *Streptococcus* and MMP3 (p = 0.007).

We observed no significant correlation between TP53 mutation status and PD-L1 expression or immune cell infiltration (Additional file 3: Figure S6). No correlation between TMB and PD-L1 expression or immune cells was observed but significant reverse correlation was evident with CD8 T cells (p = 0.02, Additional file 2: Table S13).
Fusobacterium is enriched in oral cancer

C. Michikawa et al.

**Fig. 2.** Bacterial differences in tumor and adjacent normal tissues. (A) Comparison of alpha diversity scores in adjacent normal \( (n = 36) \) and tumor \( (n = 33) \) using the Chao 1 \( (p = 0.02) \), the Shannon \( (p = 0.16) \), and Simpson \( (p = 0.18) \) indices by Mann-Whitney U (MW) test. (B) Beta-diversity using weighted UniFrac distances (left) and Bray-Curtis dissimilarity (right). (C) Association networks (Anets) of samples in terms of pair-wise similarity of shared species richness profiles. The isolated samples (bottom left) have no unique information. Nodes of the network represent samples connected by edges if they have significant pair-wise association of the shared richness profile (Pearson correlation \( R > 0.70 \)). (D) Cladogram with linear discriminant analysis (LDA) effect size (LEfSe) method to show the phylogenetic distribution of bacteria which were significantly enriched in the tumor (green) or normal (red) samples. LDA scores computed for differentially abundant taxa and the length indicates effect size associated with a taxon, \( p = 0.05 \) for the Kruskal-Wallis H statistic; LDA score \( > 2.0 \). (E) Differentially enriched bacteria at genus level and alpha-diversity indices in tumor, the Chao 1, the Shannon, and the Simpson.
Fusobacterium is enriched in oral cancer and promotes induction of programmed death-ligand 1 (PD-L1). C. Michikawa et al. Neoplasia Vol. 31, No. xxx 2022

Fig. 2. Continued
Periodontal Fusobacterium causes increased PD-L1, MYC, and ERK1 expression in head and neck SCC cell lines

In order to assess the relationship between Fusobacterium and PD-L1 expression, we screened six human head and neck SCC cell lines (HN, CAL27, BHY, FaDu, OQ01, and RPMI2650) to determine baseline PD-L1 mRNA expression relative to the PD-L1 deficient control leukemia cell line K562 using qRT-PCR [25]. OQ01 and RPMI 2650 both had significantly lower fold change of PD-L1 expression compared to the next highest cell line (FaDu), (p = 0.046 and p = 0.035 respectively, Additional file 3: Figure S7A). To confirm PD-L1 expression on the surface protein of head and neck SCC cell lines, we stained cells with anti-PDL1 mAb. Flow cytometry analysis of stained cells demonstrated high surface PD-L1 expression in HN, CAL27, BHY and FaDu, but not in K562, OQ01, and RPMI 2650 when compared to unstained control cells (Additional file 3: Figure S7B). Relative expression level of PD-L1 mRNA and surface protein was concordant among cell lines tested.

We then chose cell lines with low relative PD-L1 gene expression (OQ01 and RPMI2650) to examine the effects of periodontal bacterial strains on expression of PD-L1 and associated genes. OQ01, a primary oral tongue SCC cell line, was infected individually with four bacterial species for 24 hours; T. denticola, T. forsythia, F. periodonticum and F. vincentii. The expression level of PD-L1 mRNA was measured 24 hrs after infection using qRT-PCR as described in the Methods section. Infection with T. denticola and T. forsythia did not significantly increase PD-L1 expression relative to the uninfected control, (p = 0.926 and p = 0.498, respectively). However, OQ01 cell infection with F. periodonticum, and F. vincentii caused significant increase in relative PD-L1 expression (p = 0.0001 and p = 0.0002, respectively). We then examined the effects of F. periodonticum and F. vincentii on RPMI 2650 cells. Infection with these bacterial strains both caused significantly increased PD-L1 mRNA expression versus the uninfected control (F. periodonticum = 0.03, and F. vincentii = 0.01; Fig. 5A). We investigated mRNA expression in the MYC/ERK1 pathway in head and neck SCC cell lines after bacterial infection because this pathway is involved in PD-L1 gene expression. MYC and ERK1 expression were significantly increased in OQ01 (MYC: F. periodonticum = 0.0037 and F. vincentii = 0.0057, and ERK1; F. periodonticum = 0.0007, and F. vincentii = 0.0188) and RPMI 2650 (MYC; F. periodonticum = 0.08 (ns), and F. vincentii = 0.036, and ERK1; F. periodonticum = 0.0013, and F. vincentii = 0.0087, Fig. 5B).

To further examine the effects of periodontal Fusobacterium strains on surface PD-L1 protein expression, OQ01 and RPMI 2650 were infected individually with F. periodonticum and F. vincentii then subjected to flow cytometry. In OQ01 cells, F. periodonticum and F. vincentii infection caused significant increase in PD-L1 expression compared to uninfected controls (p = 0.003 and p = 0.004, respectively, Fig. 6A). In RPMI 2650 cells, F. vincentii caused significantly increased PD-L1 expression (p = 0.045; Fig. 6B).

Discussion

In this study, we combined 16S rRNA gene sequencing, WES, and RNA sequencing technology to examine oral bacteria in the context of tumor mutational status and immune profiling, for a single oral cancer subtype. Several studies have examined the oral SCC bacterial microbiome with 16S RNA sequencing, and many of the oral and oropharyngeal subsites have been extensively profiled [26-28]. Similar to other studies in head and neck cancer, we found that tumor and adjacent normal tissues share the majority of OTUs, and specifically belong to 5 major phyla (Firmicutes, Bacteroides, Proteobacteria, Fusobacteria, and Actinobacteria) [10, 29-31]. In our study, we did find significant differences between abundances of taxa using LEfSe. Fusobacterium was associated with oral tongue tumor samples, while Rothia and Streptococcus were enriched in adjacent normal tissues. Although this does not account for the paired nature of the data and p-values are not corrected for multiple comparisons, our findings are consistent with prior studies in which Fusobacterium was demonstrated to be significantly enriched in oral cancer samples, while Rothia and Streptococcus were enriched in adjacent normal tissues [8,9].
Fusobacterium is enriched in oral cancer and promotes induction of programmed death-ligand 1 (PD-L1) C. Michikawa et al. Neoplasia Vol. 31, No. xxx 2022

Fig. 5. mRNA expression of PD-L1 and associated genes in head and neck SCC cell lines after Fusobacterium infection. (A) PD-L1 mRNA expression with *T. denticola* and *T. forsythia* in OQ01 cell lines and with *F. periodonticum* and *F. Vincentii* in OQ01 and RPMI 2650 cell lines. (B) mRNA expression of PD-L1 pathway associated genes with the in OQ01 (left) and RPMI 2650 cells (right). Data are shown as the log2 fold change of gene expression relative to uninfected control. All bar graph results are presented as mean ± SEM based on 3 independent experiments (*p < 0.05; **p < 0.01, ***p < 0.001). Solid bars represent infection with *F. periodonticum* (FP) and bars with dots represent infection with *F. vincentii* (FV).

Fig. 6. PD-L1 surface protein expression in head and neck SCC cell lines after Fusobacterium infection. (A)% PD-L1+ cells as determined by flow cytometry after infection with *Fusobacterium* in OQ01 and RPMI 2650 cell lines. (B)Representative flow histograms for the respective cell lines. Bar graph results are presented as mean ± SEM based on 3 independent infection experiments for each cell line (*p < 0.05; **p < 0.01).
In general, the previously reported bacterial profiles relevant to head and neck cancer including oral tongue SCC have been somewhat heterogeneous and conflicting. For example, some studies have found elevated Rothia, Streptococcus, Porphyromonas, or reduced Actinomyces, Corynebacterium, or Kingella in cancer cases compared to controls [29-33]. Across the published literature, there is a wide range of differences in sample processing, subsite heterogeneity, material collected (e.g., saliva, tissue, swab, or oral wash), control selection (paired adjacent normal site or matched healthy subjects), and analysis method. It has been demonstrated that each subsite, even within oral cavity, has unique bacterial colonization [34,35]. These factors are likely to make comparisons difficult and contribute to result inconsistency. In the present study, the samples were from only one single oral cancer subsite (oral tongue), a single institution, and collection/analysis methods were kept uniform. In addition, we used adjacent normal tissues as a control so that we could adjust for inter-individual differences, which have marked variability even among healthy people [20].

The association between tumor diversity and the cancerous disease state has been investigated in a variety of cancers [15,36,37]. We demonstrated that Rothia and the species Streptococcus were associated with increased tumor alpha diversity while Fusobacterium had an inverse relationship to tumor alpha-diversity (Simpson index). The combination of decreased Rothia and Streptococcus with the corresponding increased Fusobacterium abundance, may affect the overall tumor diversity, resulting in a modest difference between tumor and normal. In pancreatic carcinoma and melanoma studies, lower alpha diversity was associated with worse overall prognosis and treatment response [15,36,37]. In our study, lower tumor alpha diversity was correlated with increased Fusobacterium, decreased Rothia and Streptococcus species, mutant TP53, and increased TMB, suggesting that lower alpha-diversity may be more mutagenic. Changes in the oral tumor microbiome compared to controls appears that, while the overall composition of bacteria in the community seems quite similar between the two groups, the relative abundance of community members may result in changes in bacterial functional pathways that can have implications on tumor cell survival/propagation (such as TP53 mutations) but the underlying mechanism is unclear. Dong et al. found an association between mutant TP53 and increased TMB, PD-L1 expression, and CD8 + T cell infiltration, in lung adenocarcinoma [38]. In our study, we observed the association between mutant TP53 and higher TMB (p = 0.0003, Additional file 3: Figure S8), but did not demonstrate an association between TP53 mutation status and PD-L1, or immune cell infiltration by RNA sequencing in oral tongue tumors.

Increased Fusobacterium was, however, significantly associated with increased PD-L1 RNA expression in patient tumor samples. We then investigated the effects of Fusobacterium infection on PD-L1 expression in head and neck cancer cell lines. We demonstrated that Fusobacterium (F. periodonticum and F. vincentii) increases PD-L1 expression in head and neck SCC cell lines that have low baseline PD-L1 expression (OQO1 and RPMI 2650). Prior investigations have demonstrated that bacterial infection with Porphyromonas gingivalis caused upregulation of PD-L1 in oral cancer cell lines (BH1Y and SCC-25) [39]. To our knowledge, this is the first study demonstrating increased PD-L1 expression in response to infection with Fusobacterium species in head and neck SCC cell lines. PD-L1, which is an immune inhibitory receptor ligand, is widely expressed on both tumor cells and tumor infiltrating immune cells including lymphocytes, macrophages, and dendritic cells in head and neck SCC [40]. Upregulation of PD-L1 on cancer cells and ligation to its receptor, programmed cell death protein 1 (PD-1) on activated T cells, is one of the important mechanisms for cancer cell escape from immune surveillance [41]. Our results suggest that tumor cell upregulation of PD-L1 in response to oral pathobionts, such as Fusobacterium, may influence immune evasion by oral carcinomas, allowing for tumor propagation. The PD-1/PD-L1 axis is an important checkpoint mediating immune resistance in head and neck SCC, as evidenced by the therapeutic benefit of PD-1 inhibitors which have been FDA approved for recurrent/metastatic head and neck cancers with promising results [42-44].

There are several mechanisms that can influence PD-L1 expression on cancer cells via both intrinsic and extrinsic signals. Intrinsic signals are from constitutive oncogenic signaling pathways within the tumor cell. Casey et al. demonstrated that the MYC oncogene can upregulate PD-L1 expression through directly binding to the promoter of the PD-L1 gene and that inhibition of MYC reduced PD-L1 expression using many tumor types [45]. The ERK cascade directly phosphorylates MYC at serine 62, resulting in enhancing MYC expression and its stability [46]. Jiang et al. demonstrated that activation of ERK signaling drives PD-L1 expression at both mRNA and protein level [47]. In our study, we demonstrated increased mRNA expression of MYC and ERK1 with infection of the same periodontal strains that also increase PD-L1 in head and neck SCC cell lines. Moreover, Rubinstein and Wang et al. revealed that F. nucleatum adhesion to E-cadherin on epithelial cells can lead to increased expression of pro-inflammatory cytokines but also variety of oncopgenes including MYC [14]. While further mechanistic studies are needed, we speculate that bacterial infection by Fusobacterium may induce the intrinsic pathway for PD-L1 expression, possibly via induction of the ERK/MYC signaling.

Extrinsic factors can also increase tumor PD-L1 expression in response to inflammatory factors secreted in the tumor microenvironment during anti-tumor responses or other inflammatory environment caused by bacteria [13,14,48–50]. Bacterial infections can set up conditions that enhance secreting pro-inflammatory cytokines and pathways including IL-6, IL-8, IL-18, NF-κB, COX-2, or MMP3 in the tumor microenvironment, which can in turn increase PD-L1 expression and promote immune suppression [13,14]. In our study, we did not demonstrate a correlation between Fusobacterium and immune cell infiltration nor the pro-inflammatory cytokines/pathways by RNA sequencing. Perhaps the inflammatory changes in the human oral cavity require a community of bacteria to set up these conditions, and limiting analysis to a single genus may not be sufficient to detect significant changes.

A potential limitation of our study could be the lack of HPV testing in our tumor samples. Given the rarity of HPV positivity and unknown clinical meaningfulness of positivity in the oral tongue subsite, [51] we did not conduct HPV testing in our cohort. Additionally, paired normal samples were not evaluated for RNA expression profiling.

**Conclusions**

Fusobacterium was found to be abundant in oral tongue cancers in our study, and may induce PD-L1 expression through a yet to be defined mechanism. With the understanding of this interaction, therapeutic targets may be designed to block effects of Fusobacterium in patients with premalignant lesions or high risk of recurrence.

**Declarations**

**Ethics approval and consent to participate**

All human studies here including sample collection and sharing the de-identified data was approved by the Institutional Review Board (LAB 08-0848 and PA12-0995) and conducted in accordance with Helsinki Declaration. Written informed consent or waiver of written informed consent were provided as part of the approval process.

**Consent for publication**

Not applicable.
Availability of data and materials

All processed data generated during this study are included in the manuscript and supplements. Raw data will be made available upon request and with appropriate Institutional Review Board approval.

Funding

This project was supported by The Kadoorie Charitable Foundation. EKLc was supported in part by the Andrew J. Semesco Foundation, Ocala, FL. AMH acknowledges financial support from UMM ALQURA University, Saudi Arabia and Saudi Arabian Cultural Mission. TVK, XW, and JZ acknowledges the Moonshot program at the University of Texas MD Anderson Cancer Center for financial support of this study. JAW is supported by the National Institutes of Health (1R01CA219896-01A1), the Melanoma Research Alliance (402202), American Association for Cancer Research Stand Up To Cancer, and the MD Anderson Melanoma Program. NLS is supported by the NIH NIDCR (1K08DE029503).

Declaration of Competing Interests

VG has consulted for MicrobiomeDX and is currently employed by AstraZeneca. VG is an inventor on US patent (PCT/US17/53,717) relating to the microbiome. VG is inventor on a provisional US patent (WO2020106983A1), JAW is an inventor on a patents WO2018064165A2, WO2019191390A2, WO2020106983A1, WO2020150429A1 that covers methods to enhance immune checkpoint blockade responses and reduce associated toxicities by modulating the microbiome. JAW also reports compensation for speaker’s bureau and honoraria from Imedex, Dava Oncology, Omniprex, Illumina, Gilead, PeerView, Physician Education Resource, MedImmune and Bristol-Myers Squibb and serves as a consultant / advisory board member for Roche/Genentech, Novartis, AstraZeneca, GlaxoSmithKline, Bristol-Myers Squibb, Merck, Biothera Pharmaceuticals. JAW also receives research support from GlaxoSmithKline, Roche/Genentech, Bristol-Myers Squibb, and Novartis. The remaining authors declare no competing interests.

CRediT authorship contribution statement

Chieko Michikawa: Data curation, Formal analysis, Investigation, Project administration, Visualization, Writing – original draft. Vancheswaran Gopalakrishnan: Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. Amani M. Harrandah: Data curation, Formal analysis, Investigation, Writing – review & editing. Tatiana V Karpinets: Formal analysis, Methodology, Visualization, Writing – review & editing. Rekha Rani Garg: Data curation, Formal analysis, Investigation, Writing – review & editing. Randy A. Chu: Project administration, Resources, Writing – review & editing. Yuk Pheel Park: Data curation, Writing – review & editing. Sasianka S. Chukkapalli: Data curation, Writing – review & editing. Nikhita Yadlapalli: Data curation, Writing – review & editing. Kelly C. Erikson-Carter: Resources, Writing – review & editing. Frederico Omar Gleber-Netto: Formal analysis, Writing – review & editing. Elias Sayour: Supervision, Writing – review & editing. Ann Progulske-Fox: Supervision, Writing – review & editing. Edward K.L. Chan: Supervision, Writing – review & editing. Xiaogang Wu: Methodology, Writing – review & editing. Jianhua Zhang: Methodology, Writing – review & editing. Curtis R. Pickering: Formal analysis, Writing – review & editing. Jennifer A. Wargo: Conceptualization, Supervision, Writing – review & editing. Jeffrey N. Myers: Conceptualization, Funding acquisition, Supervision, Writing – review & editing. Natalie Silver: Conceptualization, Formal analysis, Funding acquisition, Supervision, Writing – original draft.

Acknowledgements

The authors thank Department of Pathology for sharing the samples, Dianna B. Roberts, Bobby Banay, Deborah A. Rodriguez, Bridget E. Reeves, and Barbara deLeón for administrative support, Evelo Biosciences for their cooperation in 16S rRNA gene sequencing, Institute for Personalized Cancer Therapy (IPCT) for WES and RNA sequencing, and High-Performance Computing at the University of Texas MD Anderson Cancer Center for providing computational resources that have contributed to the results in this project.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2022.100813.

References

[1] Sklenicka S, Gardiner S, Dierks EJ, Potter BE, Bell RB. Survival analysis and risk factors for recurrence in oral squamous cell carcinoma: does surgical salvage affect outcome? J Oral Maxillofac Surg 2010;68(6):1270–5.
[2] Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011;61(2):69–90.
[3] Khan Z, Tonnes J, Muller S. Smokeless tobacco and oral cancer in South Asia: a systematic review with meta-analysis. J Cancer Epidemiol 2014;2014:394966.
[4] Harris SL, Kimple RJ, Hayes DN, Couch ME, Rosenman JG. Never-smokers, never-drinkers: unique clinical subgroup of young patients with head and neck squamous cell cancers. Head Neck 2010;32(4):499–503.
[5] Pickering CR, Zhang J, Neskey DM, Zhao M, Jasser SA, Wang J, et al. Squamous cell carcinoma of the oral tongue in young non-smokers is genomically similar to tumors in older smokers. Clin Cancer Res 2014;20(14):3842–8.
[6] Campbell BR, Chen Z, Faden DL, Agraval N, Li RJ, Hanvas GI, et al. The mutational landscape of early- and typical-onset oral tongue squamous cell carcino.
[7] Cancer 2021;127(4):544–53.
[8] Yao QW, Zhou DS, Peng HJ, Ji P, Liu DS. Association of periodontal disease with oral cancer: a meta-analysis. Tissour Biol 2014;35(7):7073–7.
[9] Al-Herih NN, Nasher AT, Maryoud MY, Hemeida HE, Chen T, Idris AM, et al. Inflammatory bacteriome featuring Fusobacterium nucleatum and Pseudomonas aeruginosa identified in association with oral squamous cell carcinoma. Sci Rep 2017;7(1):1834.
[10] Zhao H, Chu M, Huang Z, Yang X, Ran S, Hu B, et al. Variations in oral microbiota associated with oral cancer. Sci Rep 2017;7(1):11773.
[11] Schmidt BL, Kuczynski J, Bhattacharya A, Huey B, Corby PM, Queiroz EL, et al. Changes in abundance of oral microbiota associated with oral cancer. PLoS One 2014;9(6):e98741.
[12] Kilian M, Chapple IL, Hanning M, Marsh PD, Meuric V, Pedersen AM, et al. The oral microbiome - an update for oral healthcare professionals. Br Dent J 2016;221(10):657–66.
[13] Sultan AS, Kong EF, Rick AM, Jabra-Rizk MA. The oral microbiome: a lesson in coexistence. PLoS Pathog 2018;14(1):e1006719.
[14] Kostic AD, Chun E, Robertson L, Glickman JN, Gallini CA, Michaud M, et al. Fusobacterium nucleatum potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. Cell Host Microbe 2013;14(2):207–15.
[15] Rubinstein MR, Wang X, Liu W, Hao Y, Cai G, Han YW. Fusobacterium nucleatum promotes colorectal carcinogenesis by modulating E-cadherin/beta-catenin signaling via its FadA adhesion. Cell Host Microbe 2013;14(2):195–206.
[16] Gopalakrishnan V, Spencer CN, Nezi L, Reuben A, Andrews MC, Karpinets TV, et al. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. Science 2018;359(6371):97–103.
[17] Andrews MC, Duong CPM, Gopalakrishnan V, Ichiba V, Chen WS, Derosa L, et al. Gut microbiota signatures are associated with toxicity to combined CTLA-4 and PD-1 blockade. Nat Med 2021.
Neoplasia

Park YP, Jin L, Bennett KB, Wang D,Fredenburg KM, Tseng JE, et al. CD70 as a target for chimeric antigen receptor T cells in head and neck squamous cell carcinoma. *Oncof Oncol 2018;7(8):145–50.

Chukkapalli SS, Velsko IM, Rivera-Kweh MF, Zheng D, Lucas AR, Kesavalu L. Polymicrobial oral infection with four periodontal bacteria orchestrates a distinct inflammatory response and atherosclerosis in ApoE null mice. *PLoS One 2015;10(11):e0143291.

Harrandah AM, Chukkapalli SS, Bhattacharyya I, Progulske-Fox A, Chan EKL. Fusobacteria modulate oral carcinogenesis and promote cancer progression. *J Oral Microbiol 2020;13(1):1849493.

Human Microbiome Project C. Structure, function and diversity of the healthy human microbiome. *Nature 2012;486(7402):207–14.

Louzupone C, Knight R. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol 2005;71(12):8228–35.

McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbial community data. *PLoS One 2013;8(4):e61217.

Karpineti TV, Gopalakrishnan V, Wargo J, Futreal AP, Schadt CW, Zhang J. Linking Associations of Rare Low-Abundance Species to Their Environments by Associations networks. *Front Microbiol 2018:9.297.

Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. *Genome Biol 2011;12(6):R60.

Benson DM Jr, Baken CE, Mishra A, Hofmeister CC, Efereba Y, Becknell B, et al. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood 2010;116(9):2286–94.

Chattonpadhyay I, Verma M, Panda M. Role of oral microbiome signatures in diagnosis and prognosis of oral cancer. *Technol Cancer Res Treat 2019;18:153033819873943.

Sun J, Tang Q, Yu S, Xie M, Xie Y, Chen G, et al. Role of the oral microbiota in cancer evolution and progression. *Cancer Med 2020;9(17):6306–21.

Shillitoe EJ. *The Microbiome of Oral Cancer. Crit Rev Oncov 2018;23(3–4):153–60.

Pushalkar S, Mane SP, Ji X, Li Y, Evans C, Crasta OR, et al. Microbial diversity in saliva of oral squamous cell carcinoma. *FEMS Immunol Med Microbiol 2011;61(3):269–77.

Wang H, Funchain P, Bebek G, Altemus J, Zhang H, Niazi F, et al. Microbiome differences in tumor and paired-normal tissue in head and neck squamous cell carcinomas. *Genome Biol 2017;9(1):14.

Hayes RB, Ahn J, Fan X, Peters BA, Ma Y, Yang L, et al. Association of oral microbiome with risk for incident head and neck squamous cell cancer. *JAMA Oncol 2018;4(3):358–65.

Banerjee S, Tian T, Wei Z, Peck KN, Shih N, Chalian AA, et al. Microbial signatures associated with oropharyngeal and oral squamous cell carcinomas. *Sci Rep 2017;7(1):4036.

Pushalkar S, Ji X, Li Y, Estilo C, Yegnanarayana R, Singh B, et al. Comparison of oral microbiota in tumor and non-tumor tissues of patients with oral squamous cell carcinoma. *BMIC Microbiol 2012;12:144.

Mager DL, Ximenex-Fyvie LA, Halfajee AD, Socransky SS. Distribution of selected bacterial species on intraoral surfaces. *J Clin Periodontol 2003;30(7):644–54.

Segata N, Haake SK, Mannan P, Lemon KP, Waldron L, Gevers D, et al. Composition of the adult digestive tract bacterial microbiome based on seven mouth surfaces, tonsils, throat and stool samples. * Genome Biol 2012;13(6):R42.