NEOPLASIA
www.neoplasia.com

Volume 31 Number xxx Month 2022 pp. 100813

Original Research

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

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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 subsite. Here, paired tumor and adjacent normal tissues 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.

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

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 $p \leq 0.05$. 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 ug/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 (F.) nucleatum* ssp. *vincentii (F. vincentii), (*ATCC, 49256), *F. periodonticum* (ATCC, 33693), *Treponema denticola* (*T. denticola*), (ATCC, 35404) and *Tannerella 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 (OD_{550nm}). Culture characteristics and gram stains were assessed for all cultures prepared in this study.

Bacterial Infection of cell lines

Cancer cells were seeded at 5×10^5/mL in 6 well plates at 37°C overnight in antibiotic free medium. Bacteria was 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 ($2^{\wedge -\Delta\Delta CT}$) 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 * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$.

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 fixed 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 $p \leq 0.05$.

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 have 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.

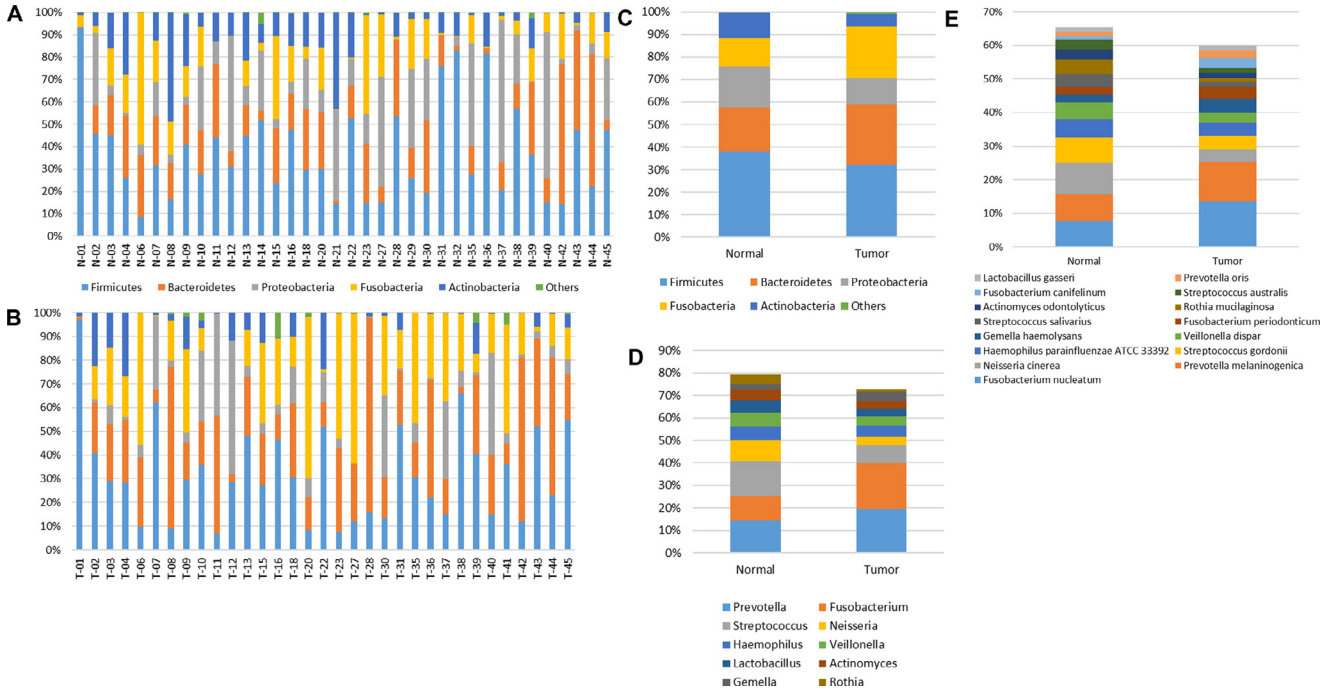

Fig. 1. Bacterial Profiling. (A) Individual relative abundance of top 5 phyla in adjacent normal ($n = 36$) and (B) tumor ($n = 33$) tissues. (C) Average relative abundance of phyla, (D) genera, and (E) species. In total, 15 phyla, 252 genera, and 492 species were found in all samples.

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 *Fusobacerium* 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 *Streptococccus* 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)

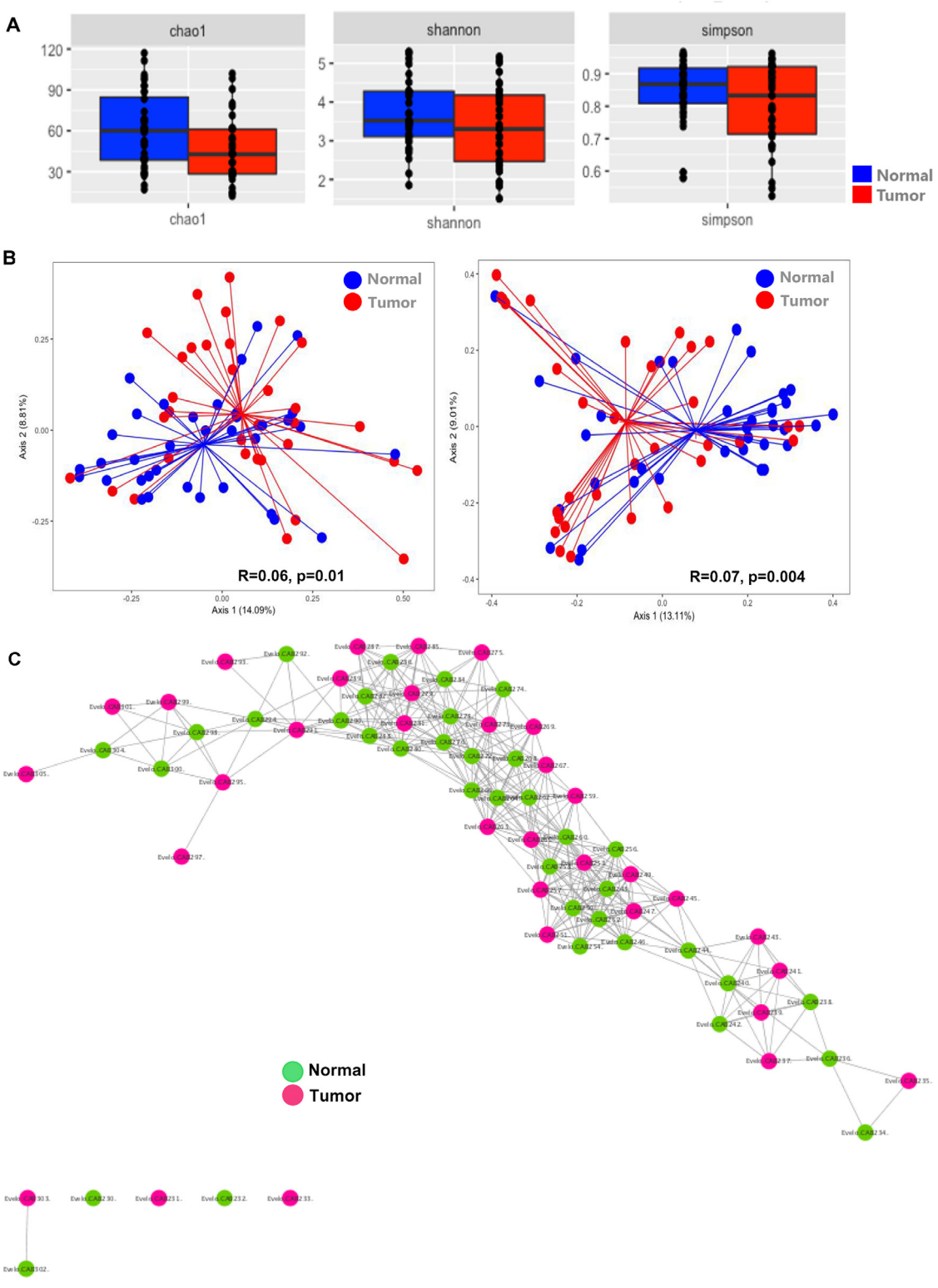

Fig. 2. Bacterial differences in tumor and adjacent normal tissues. (A) Comparison of alpha diversity scores in adjacent normal ($n = 36$) and tumor ($n = 33$) using the Chao 1 ($p = 0.02$), the Shannon ($p = 0.16$), and Simpson ($p = 0.18$) indices by Mann-Whitney U (MW) test. (B) Beta-diversity using 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.

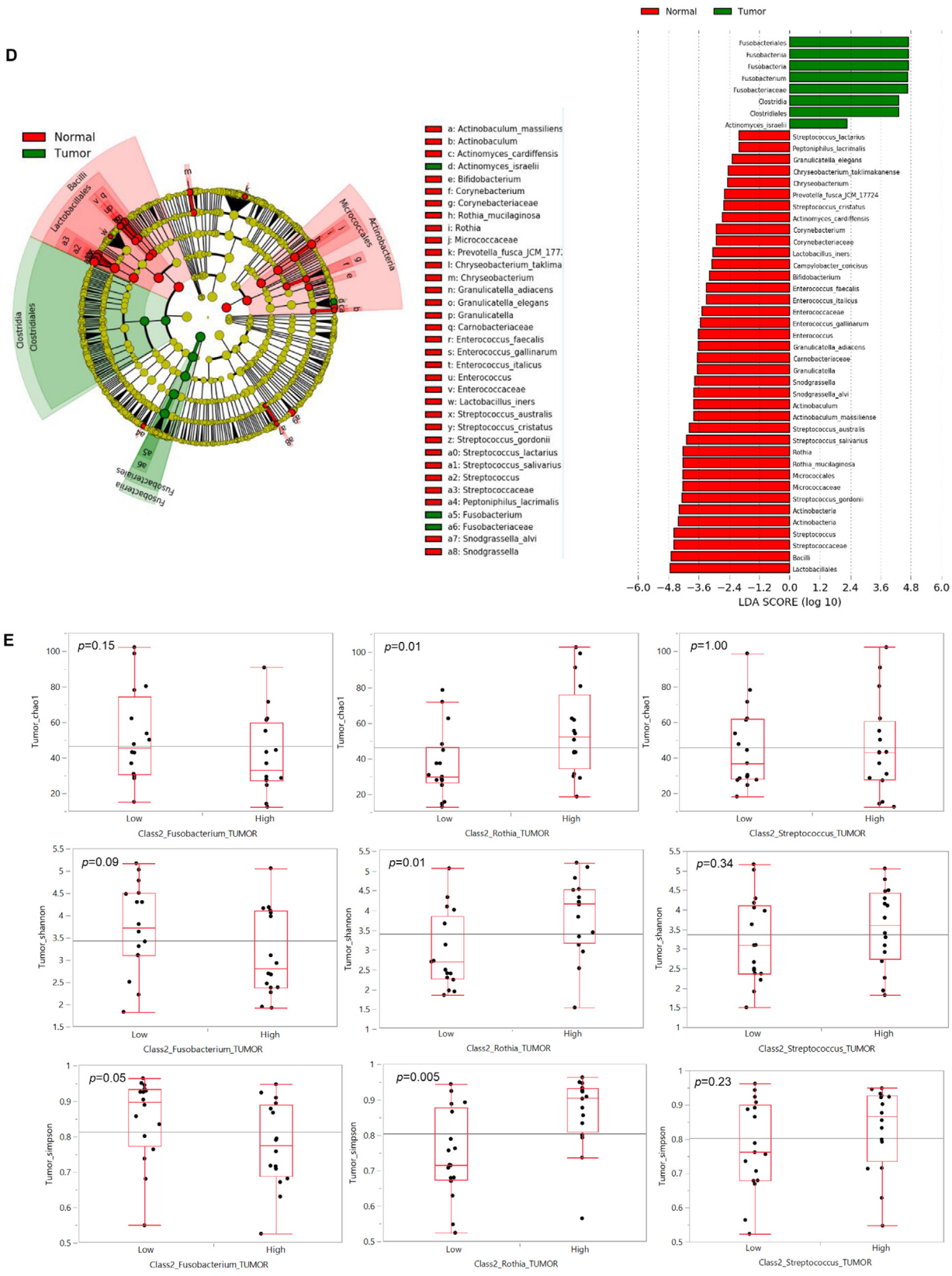

Fig. 2. Continued

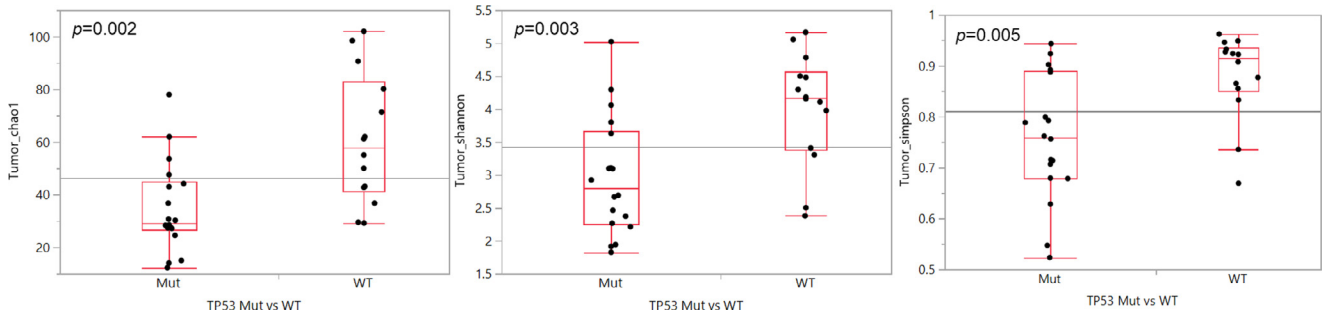

Fig. 3. Association with TP53 status and alpha-diversity in tumor. Mut, mutation; WT, wild-type.

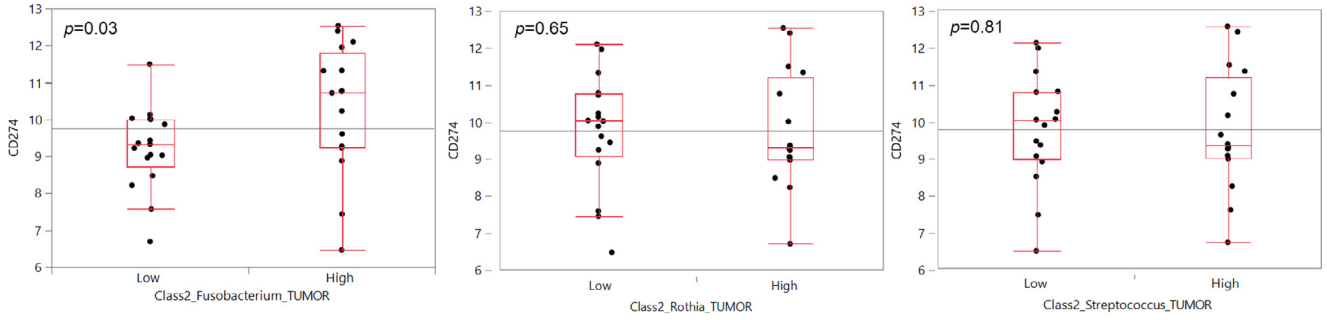

Fig. 4. Differentially enriched bacteria and PD-L1 (CD274) expression.

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. periodondicum* 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. periodonticump* = 0.03, and *F. vincentiip* = 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. periodonticump* = 0.0037 and *F. vincentiip* = 0.0057, and ERK1; *F. periodonticump* = 0.0007, and *F. vincentiip* = 0.0188) and RPMI 2650 (MYC; *F. periodonticump* = 0.08 (ns), and *F. vincentiip* = 0.036, and ERK1; *F. periodonticump* = 0.0013, and *F. vincentiip* = 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 subsite. 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].

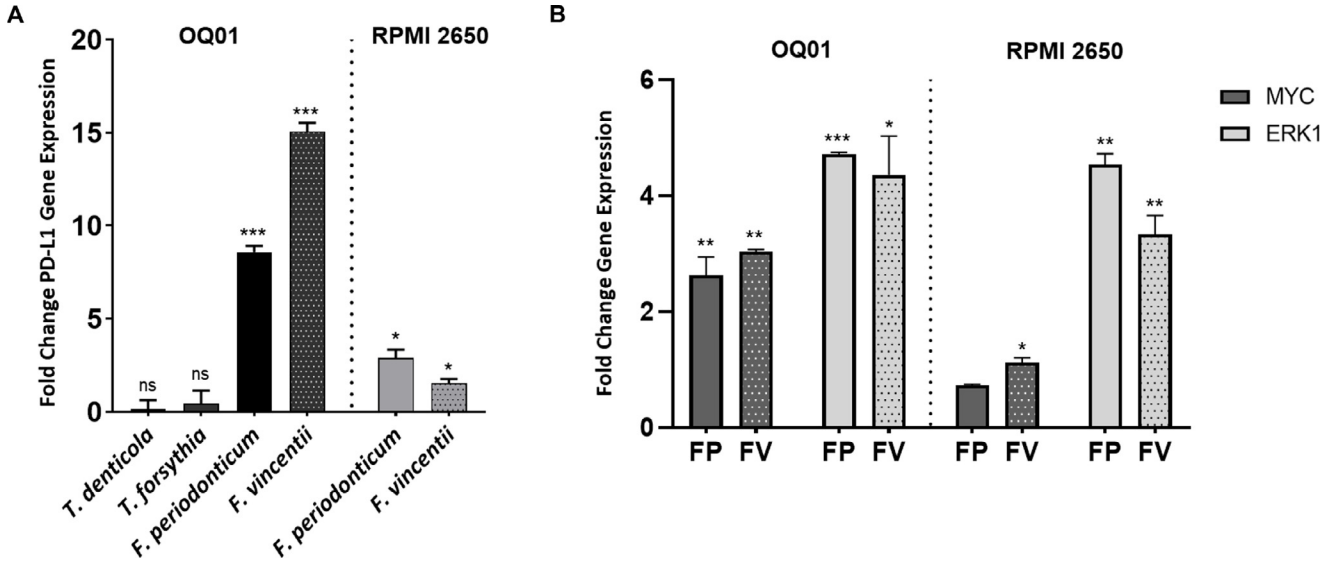

Fig. 5. mRNA expression of PD-L1 and associated genes in head and neck SCC cell lines after *Fusobacterium* infection. (A) PD-L1 mRNA expression with *T. denticola* and *T. forsythia* in OQ01 cell lines and with *F. periodonticum* and *F. Vincentii* in OQ01 and RPMI 2650 cell lines. (B) mRNA expression 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).

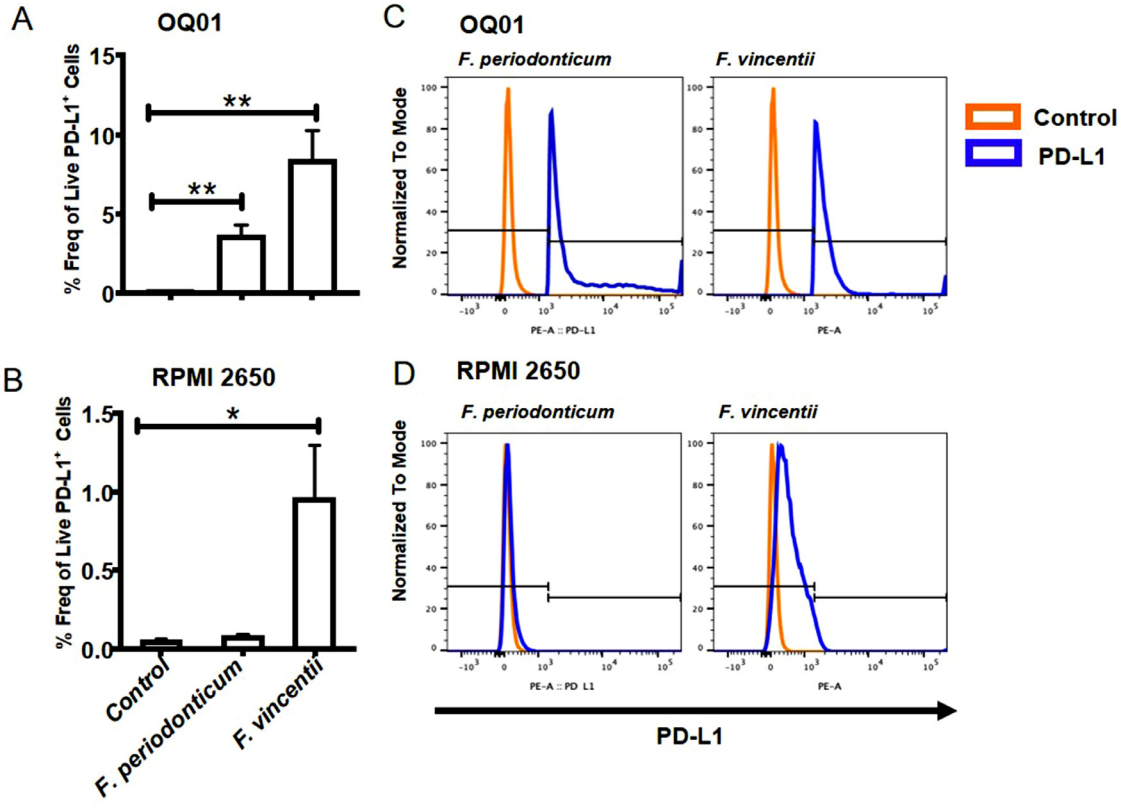

Fig. 6. PD-L1 surface protein expression in head and neck SCC cell lines after *Fusobacterium* infection. (A)% PD-L1+ cells as determined by flow cytometry after infection with *Fusobacterium* in OQ01 and RPMI 2650 cell lines. (B)Representative flow histograms for the respective cell lines. Bar graph 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, Streptococccus, Parvimonas*, 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 (BHY 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 oncogenes 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 (4022024), American Association for Cancer Research Stand Up To Cancer, and the MD Anderson Melanoma Moonshot 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. **Sasanka S. Chukkapallia:** 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. **Christian Jobin:** Supervision, Writing – review & editing. **Jennifer A. Wargo:** Conceptualization, Supervision, Writing – review & editing. **Curtis R. Pickering:** Formal analysis, 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 deLeon 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.

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