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Original Article

ORIGINAL ARTICLE

The interplay between oral microbiome, lifestyle factors and genetic polymorphisms in the risk of oral squamous cell carcinoma

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Abstract

Poor oral hygiene may lead to overgrowth of pathogenic oral bacteria, which may induce chronic inflammation to promote the oncogenesis of oral squamous cell carcinoma (OSCC). This study investigated the association between oral bacterial profile and OSCC risk in a case–control study of 138 OSCC cases and 151 controls (88 cases and 90 controls for the discovery group and 50 cases and 61 controls for the validation group). Oral bacterial profiles were characterized by targeted sequencing of the 16S rRNA gene. Three species of periodontopathogenic bacteria, *Prevotella tannerae*, *Fusobacterium nucleatum*, *and Prevotella intermedia*, were associated with an increased OSCC risk. This association was modified by the genetic polymorphisms of *TLR2* and *TLR4*. Use of alcohol, betel quids and cigarettes and poor oral hygiene were associated with a higher percentage of oral periodontopathogenic bacteria. The association between alcohol and periodontopathogenic bacteria was modified by the genetic polymorphism of *ALDH2*, with a stronger positive association observed among the ALDH2-deficient individuals. The percentage of periodontopathogenic bacteria was positively correlated with the level of salivary IL1β, an inflammatory cytokine. Overall, our results showed a positive association between periodontopathogenic bacteria and OSCC risk and this relationship may be influenced by lifestyle and genetic factors. Our results provided further biological support for the established association between poor oral hygiene and OSCC risk. This suggested that improving oral hygiene may reduce OSCC risk and should be part of a public health campaign to prevent the occurrence of OSCC.

Introduction

Approximately 300 000 cases of oral cancer are diagnosed worldwide annually (1). More than 90% of the oral cancers are oral squamous cell carcinoma (OSCC) and the remaining rare histologic subtypes include minor salivary gland carcinomas,

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Abbreviations

AUC	area under the curve
CI	confidence interval
OR	odds ratio
OSCC	oral squamous cell carcinoma
OTU	operational taxonomic unit
ROC	receiver operating characteristic curve
SNP	single nucleotide polymorphism
TLR	Toll-like receptor.

lymphomas and melanoma (2,3). The majority of oral cancers can be attributed to the use of alcohol, betel quids and cigarettes (2). However, most users of alcohol, betel quids and cigarettes will not develop oral cancer in their lifetime, suggesting the contribution of other factors.

Accumulating evidence strongly supports poor oral hygiene as an independent risk factor of oral cancer. An increased oral cancer risk has been consistently associated with infrequent tooth brushing (4–14). The majority of published studies reported a positive association between no regular dental visit and oral cancer (6,11,13–19). Poor oral hygiene may result in periodontitis. Many studies have examined the association between oral cancer and periodontitis either directly or indirectly with indicator variables, including gum bleeding and tooth loss. Among the nine studies investigating the association between gum bleeding and oral cancer, six reported a positive association (6,9–11,18,20). In a meta-analysis of seven case–control studies, a linear positive dose response was observed between the number of tooth lost and oral cancer risk (21). Three studies examining the association between periodontitis and oral cancer all observed a strong positive association (22–24).

Poor oral hygiene may lead to overgrowth of the pathogenic oral bacteria and induce chronic inflammation to promote the oncogenesis of oral cancer. In the oral mucosa, Toll-like receptors (TLRs) are expressed by dendritic cells and the basal layer of the squamous oral mucosal epithelium. The binding of pathogen-associated molecular patterns on bacteria to the TLRs can elicit downstream signaling to induce inflammation. Normally, oral microorganisms are kept in check by the dendritic cells (25). When the integrity of oral epithelium is breached by invasive pathogenic oral bacteria or by the use of alcohol or cigarettes, oral bacteria may bind to the TLRs on the oral basal epithelium, resulting in overt inflammation (25).

Studies have increasingly recognized the roles of microorganisms in modulating and maintaining the biological functions of the human host. When the balanced relationship between microorganisms and the human host is disrupted (dysbiosis), whether by external agents (e.g. use of alcohol and cigarettes) or by infection with pathogenic microorganisms, the risk of many diseases, including cancer, may increase (26). The current study investigated the association between oral bacterial profile and OSCC risk. In addition, the interaction between oral bacterial profile and genetic or lifestyle factors of the human host on OSCC risk was explored. Finally, we evaluated whether oral bacteria profile could be influenced by lifestyle factors, including oral hygiene and use of alcohol, betel quids and cigarettes.

Materials and methods

This study was approved by the institutional review boards of the National Health Research Institutes and the National Cheng Kung University Hospital. A signed informed consent was obtained from each study participant.

Study subjects

The study subjects in this analysis were from an ongoing case–control study of head and neck cancer. Subject recruitment started on 1 September 2010 at the Department of Otolaryngology and the Department of Stomatology at the National Cheng Kung University Hospital. The eligibility criteria for the cases were: (i) diagnosis of pathologically confirmed squamous cell carcinoma located in the oral cavity, oropharynx, hypopharynx and larynx (ICD-10 codes: C00-C10, C12-C14, C32); (ii) no previous cancer diagnosis; (iii) age ≧20 years; (iv) Han ethnicity and (v) ability to provide informed consent. Controls were frequency-matched to cases on sex and age (±5 years). The eligibility criteria for the controls were: (i) diagnosis of non-cancerous conditions of the head and neck that are not related to the use of alcohol, betel quids and cigarettes and required surgery; (ii) no previous cancer diagnosis; (iii) age ≧20 years; (iv) Han ethnicity and (v) ability to provide informed consent.

The saliva collection for characterizing oral bacterial profiles began in November 2013. The current study included only male OSCC cases and controls. The subjects were divided into the discovery group (88 cases and 90 controls) and the validation group (50 cases and 61 controls).

Data collection by in-person interview

Each participant was interviewed to collect information on age, education, use of alcohol, betel quids and cigarettes, and oral hygiene. An ever regular alcohol drinker was defined as an individual who had drunk at least once per week. An ever betel quid chewer was defined as an individual who had chewed betel quid daily for 6 consecutive months or more in his lifetime. An ever cigarette smoker was an individual who had smoked more than 100 cigarettes in his lifetime. For oral hygiene, each study subject was asked about: (i) regular dental visits (yes/no and frequency); (ii) tooth brushing (number of times per day) and (iii) use of dental floss (yes/no).

Biological specimen collection and processing

For profiling of oral bacteria, saliva sample was collected from each participant using Omnigene-ORAL (OM505) (DNA Genotek Inc., Ottawa, Ontario, Canada). Before saliva collection, each study subject was instructed to refrain from drinking, smoking, eating or chewing gum for at least 30 min. DNA was extracted from saliva within 1 week of collection using a DNA extraction kit (QIAGEN QIAamp MinElute Virus Spin Kit, QIAGEN, Venlo, Netherlands). Saliva DNA samples were stored in –80°C refrigerator until ready to use.

To measure salivary cytokines, saliva sample was initially collected in a 50 ml centrifuge tube and subsequently transferred to an 1.5 ml eppendorf tube for centrifugation at 15 000*g* for 15 min. The supernatant was then stored in the –80°C refrigerator until ready to use.

For genotyping, peripheral blood was collected from each study participant in a vacutainer tube containing ethylenediaminetetraacetic acid. The blood was then centrifuged to separate out the buffy coat. A genomic DNA purification kit (Wizard Genomic DNA Purification Kit, Promega, Madison, WI) was used to extract genomic DNA from the buffy coat. Genomic DNA samples were then stored in the –80°C refrigerator until ready to use.

Profiling of oral bacteria

Characterization of oral bacterial profile was based on targeted sequencing of 16S rRNA gene. Each run of the experiment included approximately half cases and half controls to minimize the impact of batch effect. PCR amplification of the V3-V5 regions of the bacterial 16S rRNA gene was performed using the pair of primers 341F/926R (27,28). For compatibility with Illumina MiSeq System, overhang adapter sequences (in unbolded letters) were added to the primers (in bold letters): 16S-v3-341-F: TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; 16S-v5-926-R: GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCGTCAA TTYYTTTRAGT. PCR amplification was conducted with HiFi PCR Master Mix (KAPA Biosystems, Wilmington, MA) to generate a single amplicon of approximately 700 bp. Each run of the PCR included a negative control with ddH_2O to detect background contamination. The amplicon library was purified with Agencourt AMpure XP Magnetic Beads (Beckman Coulter, Brea, CA). Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA) was used to quantify the concentration of the amplicon library. The

size distribution of the amplicon library was checked by Caliper LabChip (PerkinElmer, Waltham, MA). In addition, all PCR products were examined with gel electrophoresis to ensure the presence of the 700 bp amplicons.

Targeted sequencing of 16S rRNA gene was performed on Illumina MiSeq System (Illumina, San Diego, CA, USA). Sequences with quality score (Phred quality score) <30 (probability of incorrect base call = 1 in 1000 or 99.9%) were removed. From the saliva samples of the 289 subjects in the study, a total of 77 595 460 high-quality bacterial 16S rRNA sequence reads were obtained. Then number of reads were similar between OSCC cases and controls (mean = 270 449 reads for OSCC cases versus 266 712 reads for controls, $P = 0.80$). Related sequences were grouped into operational taxonomic units (OTUs). The grouping of the related sequences at the species level was achieved by the sharing of 97% sequence identity (29). Taxonomic classification of OTUs was performed by sequence alignment with Greengenes 16S rRNA database (30,31), version 13.5, using ClassifyReads function of the Illumina 16S Metagenomics App (Illumina). ClassifyReads is a high-performance implementation of the Ribosomal Database Project (RDP) Classifier (32). The original RDP classifier can only classify the OTUs to the genus level whereas ClassifyReads, with an improved algorithm (details available on https://support.illumina.com/content/dam/illumina-support/documents/documentation/software_documentation/basespace/16s-metagenomics-user-guide-15055860-a.pdf), could classify the OTUs to the species level with an accuracy of 98.24% (33). For each saliva sample, an oral bacterial profile was generated by summarizing the OTU counts into percentages to indicate the relative abundance of each bacterial taxon.

Genotyping of single nucleotide polymorphisms

Genotyping of five single nucleotide polymorphisms (SNPs), *ADH1B* rs1229984, *ALDH2* rs671, *TLR2* rs3804099, *TLR2* rs3804100 and *TLR4* rs11536889, was performed using Taqman-based allelic discrimination method on an Applied Biosystems 7500 Real-Time Polymerase Chain Reaction System (Applied Biosystems, Foster City, CA). For quality control, 10% of the samples were randomly selected for duplicate genotyping and the duplicates were 100% concordant. All SNPs were in Hardy–Weinberg equilibrium.

Measurement of salivary cytokines

Salivary cytokines and chemokines were measured using Milliplex map Human TH17 Magnetic Bead Panel (EMD Millipore Corporation, Billerica, MA) on Luminex 200 system (Luminex, Austin, TX). We were particularly interested in IL1β, IL6 and TNFα because these three pro-inflammatory cytokines have been consistently associated with oral cancer (34–37). For completeness, we also included other cytokines and chemokines on the panel in our analysis, including CCL20, IFNγ, IL2, IL5, IL9, IL10, IL12p70, IL13, IL15, IL17A, IL21 and IL33. Some cytokines, including IL4, IL17E, IL17F, IL22, IL23, IL27, IL28A, IL31 and TNFβ, were excluded from our analysis due to low detection levels.

Statistical analysis

The distributions of sex, age, education, consumption of alcohol, betel quids and cigarettes, and dental care habits were compared between cases and controls in the discovery and validation groups using *t*-tests (for continuous variables) and chi-squared tests (for categorical variables).

T-tests were used to compare the percentage of individual bacterial species between cases and controls in the discovery group. The analyses were limited to those species with a relative abundance of ≧1% among either the cases or controls. The species that reached statistical significance after Bonferroni correction were compared between the cases and the controls in the validation group.

Bacterial species that were successfully validated were further analyzed using data from the discovery and validation groups combined. Unconditional logistic regression was performed to estimate the odds ratio (OR) and 95% confidence interval (CI) for the association between the percentage of bacteria and OSCC risk. The percentage of bacteria was analyzed: (i) as a dichotomous variable with the median among the controls as the cutoff and (ii) as a continuous variable. To evaluate the influence of alcohol, betel quids and cigarettes on the association between oral bacteria and OSCC, analyses on the association between OSCC and oral bacteria were performed stratified by the use of alcohol, betel quids or cigarettes. Heterogeneity between the strata was evaluated by log-likelihood ratio test comparing the unconditional logistic regression model with a product term (oral bacteria × alcohol or betel quids or cigarettes) to that without a product term. The interaction between oral bacteria and *TLR* polymorphisms on OSCC risk was evaluated by analyzing the association between oral bacteria and OSCC stratified by the genotypes of *TLR2* rs3804099 and rs3804100 and *TLR4* rs11536889. The interaction was assessed by log-likelihood ratio test comparing the unconditional logistic regression model with a product term (oral bacteria × *TLR2* rs3804099 or *TLR2* rs3804100 or *TLR4* rs11536889) to that without a product term.

Two risk prediction models were compared to evaluate whether adding oral bacteria information may increase the prediction accuracy of OSCC. The first model included age, education, and consumption of alcohol, betel quids and cigarettes. In the second model, in addition to the variables contained in model 1, data on oral bacteria were also added. To evaluate the prediction accuracy of a model, a receiver operating characteristic (ROC) curve was constructed and area under the curve (AUC) was calculated using c-statistic. The two AUCs were compared using the nonparametric approach proposed by Delong *et al.* (38).

Data of the control subjects were analyzed to evaluate the factors, including alcohol drinking, betel quid chewing, cigarette smoking and poor oral hygiene, that may influence the percentage of oral bacteria using *t*-tests. We further stratified the data by *ADH1B* and *ALDH2* genotypes to investigate whether the relationship between alcohol and oral bacteria might differ according to the *ADH1B* and *ALDH2* genotypes, which may influence the efficiency of ethanol metabolism. For oral hygiene, each dental care behavior (frequency of tooth brushing, use of dental floss, and regular dental visit) was analyzed separately for its association with oral bacteria using *t*-tests. In addition, a dental care score was created by combining the three dental care behaviors. The dental care score = tooth brushing + use of dental floss + regular dental visit, with tooth brushing: ≧2 times per day = 0, <2 times per day = 1; use of dental floss: yes = 0, no = 1 and regular dental visit: yes = 0, no = 1. A dental care score of 0 or 1 = good oral hygiene, whereas 2 or 3 = poor oral hygiene. The percentages of oral bacteria were then compared between those with good oral hygiene to those with poor oral hygiene using *t*-tests.

The correlation between oral bacteria and salivary cytokines was evaluated by Pearson's correlation and Spearman's correlation. The level of each cytokine was natural log-transformed for the correlation analysis.

Results

Characteristics of the study subjects

This analysis included 88 OSCC cases and 90 controls for the discovery group and 50 OSCC cases and 61 controls for the validation group. Cases and controls were similar in mean age for both the discovery and validation groups (Supplementary Table 1, available at *Carcinogenesis* Online). For both groups, cases tended to have fewer years of education and were more likely to use alcohol, betel quids and cigarettes and have poorer oral hygiene compared to the controls. The participants in the discovery and validation groups were generally similar except that the discovery cases and the validation cases showed a significant difference in the distribution of educational level.

Comparing the relative abundance of oral bacteria between OSCC patients and controls

The sequences of approximately 2000 species of bacteria were identified from the saliva samples of the discovery group. After excluding those species with a relative abundance of <1%, 20 species of bacteria were included for case–control comparison (Table 1). After accounting for multiple comparison by Bonferroni correction (P needed to be smaller than 0.05/20 = 0.0025), four species of bacteria reached statistical significance. Among them, *Prevotella tannerae*, *Fusobacterium nucleatum and Prevotella intermedia* showed a significantly higher relative abundance and

Table 1. Comparison of relative abundance of oral bacteria between OSCC patients and controls

	Discovery group			Validation group			Discovery group + validation group		
	Case, N = 88	Control, N = 90		Case, N = 50	Control, N = 61		Case, N = 138	Control, N = 151	
Species	% (SE)	% (SE)	P value[a]	% (SE)	% (SE)	P value[a]	% (SE)	% (SE)	P value[a]
Prevotella tannerae	1.20 (0.16)	0.49 (0.07)	0.000075	0.98 (0.31)	0.32 (0.06)	0.02	1.12 (0.15)	0.42 (0.05)	0.000009
Fusobacterium nucleatum	1.05 (0.12)	0.57 (0.06)	0.0003	0.48 (0.08)	0.24 (0.04)	0.005	0.84 (0.08)	0.43 (0.04)	0.000008
Prevotella intermedia	2.61 (0.43)	0.91 (0.19)	0.0004	1.37 (0.41)	0.80 (0.16)	0.17	2.16 (0.32)	0.86 (0.13)	0.0001
Streptococcus tigurinus	2.67 (0.22)	4.02 (0.32)	0.0006	5.83 (0.54)	5.24 (0.48)	0.41			
Veillonella atypica	1.89 (0.20)	2.77 (0.22)	0.0031						
Streptococcus infantis	1.53 (0.21)	2.34 (0.18)	0.0041						
Prevotella oris	1.01 (0.24)	0.37 (0.04)	0.009						
Fusobacterium periodonticum	0.79 (0.11)	1.22 (0.15)	0.0207						
Prevotella melaninogenica	5.91 (0.54)	7.62 (0.61)	0.0389						
Neisseria lactamica	4.51 (0.75)	2.76 (0.41)	0.0396						
Veillonella dispar	0.82 (0.11)	1.22 (0.16)	0.0413						
Neisseria mucosa	4.30 (0.53)	5.96 (0.66)	0.0541						
Prevotella histicola	1.82 (0.29)	2.54 (0.26)	0.0624						
Porphyromonas gingivalis	1.64 (0.32)	0.96 (0.18)	0.0634						
Haemophilus parainfluenzae	1.18 (0.15)	1.54 (0.12)	0.0653						
Streptococcus pseudopneumoniae	4.36 (0.42)	5.45 (0.47)	0.0831						
Streptococcus oralis	0.86 (0.09)	1.04 (0.11)	0.2148						
Neisseria subflava	1.16 (0.34)	1.66 (0.43)	0.3556						
Prevotella pallens	1.17 (0.19)	1.07 (0.12)	0.6315						
Neisseria flavescens	0.95 (0.24)	1.01 (0.23)	0.855						

[a]P values were generated using T-tests.

Streptococcus tigurinus showed a lower relative abundance among the cases compared to the controls. Results of the *P. tannerae* and *F. nucleatum* were successfully validated (validation $P < 0.05$) and they were included for further analysis. Although the difference in the relative abundance of *P. intermedia* did not reach statistical significance in the validation analysis, it was included for further analysis for two reasons: (i) the difference in the relative abundance of *P. intermedia* in the validation group was in the same direction as that in the discovery group, and the non-statistical significance was partly due to a smaller sample size of the validation group and (ii) *P. intermedia*, *P tannerae* and *F. nucleatum* are all periodontopathogenic bacteria associated with periodontitis (39–41). The result of *S. tigurinus* was not validated ($P = 0.41$) and in addition, the results in the discovery group and the validation group were in the opposite directions. Therefore, *S. tigurinus* was excluded from further analysis.

Evaluating the OSCC risk associated with the three periodontopathogenic bacteria

We combined the data from the discovery and validation groups to evaluate the OSCC risk associated with the three periodontopathogenic bacteria (*P. tannerae*, *F. nucleatum* and *P. intermedia*) (Table 2). After adjusting for age, education, and use of alcohol, betel quids and cigarettes, all three periodontopathogenic bacteria were individually associated with an increased OSCC risk. When the three bacteria were combined, those with a relative abundance above the median had a 2.3 times increase in OSCC risk (OR = 2.34, 95% CI: 1.28–4.26). Every 1% increase in the total percentage of the periodontopathogenic bacteria was associated with a 28% increase in OSCC risk (OR = 1.28, 95% CI: 1.13–1.44).

The association between the periodontopathogenic bacteria and OSCC risk did not differ significantly (*P*-heterogeneity >

Table 2. The association between periodontopathogenic bacteria and OSCC risk

Bacteria	Case, *N* = 138 *n* (%)	Control, *N* = 151 *n* (%)	Crude OR (95% CI)	*P* value	Adjusted OR (95% CI)[a]	*P* value
Prevotella tannerae						
Below median (0–0.2349%)	47 (34.1)	75 (49.7)	Reference		Reference	
Above median (0.235% or more)	91 (65.9)	76 (50.3)	1.91 (1.19–3.07)	0.008	2.15 (1.21–3.82)	0.009
Every 1 %			2.22 (1.56–3.16)	<0.0001	2.22 (1.49–3.33)	<0.0001
Fusobacterium nucleatum						
Below median (0–0.299%)	45 (32.6)	75 (49.7)	Reference		Reference	
Above median (0.30% or more)	93 (67.4)	76 (50.3)	2.03 (1.27–3.29)	0.004	1.94 (1.10–3.42)	0.02
Every 1 %			2.42 (1.57–3.73)	<0.0001	1.95 (1.21–3.13)	0.006
Prevotella intermedia						
Below median (0–0.2969%)	57 (41.3)	75 (49.7)	Reference		Reference	
Above median (0.297% and more)	81 (58.7)	76 (50.3)	1.40 (0.88–2.23)	0.15	0.96 (0.54–1.73)	0.90
Every 1 %			1.25 (1.10–1.43)	0.0008	1.22 (1.06–1.40)	0.006
Periodontopathogenic bacteria[b]						
Below median (0–1.099%)	33 (23.9)	75 (49.7)	Reference		Reference	
Above median (1.1% and more)	105 (76.1)	76 (50.3)	3.14 (1.90–5.20)	<0.0001	2.34 (1.28–4.26)	0.006
Every 1 %			1.33 (1.19–1.49)	<0.0001	1.28 (1.13–1.44)	<0.0001

[a]OR and 95% CI were calculated using unconditional logistic regression, adjusted for age, education, and consumption of alcohol, betel quids and cigarettes.
[b]Sum of *Prevotella tannerae* + *Fusobacterium nucleatum* + *Prevotella intermedia*.

0.05) by the consumption of alcohol, betel quids and cigarettes (Supplementary Table 2, available at *Carcinogenesis* Online). Thus, regardless of the use of alcohol, betel quids and cigarettes, periodontopathogenic bacteria are an independent risk factor of OSCC.

ROC curve and AUC for evaluating the improvement in OSCC prediction with the addition of the periodontopathogenic bacteria variable

In Supplementary Figure 1 (available at *Carcinogenesis* Online), ROC curve 1 was constructed based on the prediction model 1 that included age, education, and use of alcohol, betel quids and cigarettes. ROC curve 2 was constructed based on the prediction model that included all of the variables in model 1 plus the periodontopathogenic bacteria variable. The AUC for ROC curve 1 was 0.778 and with the addition of the periodontopathogenic bacteria variable in ROC curve 2, the AUC improved to 0.825. The two AUCs were significantly different with $P = 0.01$.

Evaluating the interaction between periodontopathogenic bacteria and polymorphisms of *TLR2* and *TLR4* on OSCC risk

Because periodontopathogenic bacteria my induce inflammation by interacting with the TLRs, particularly TLR2 and TLR4 (42), we decided to evaluate the interaction between the SNPs of *TLR2* (rs3804099 and rs3804100) and *TLR4* (rs11536889) and periodontopathogenic bacteria on OSCC risk (Table 3). No significant interaction was detected between periodontopathogenic bacteria and each SNP on OSCC risk. A significant interaction (P interaction = 0.01) was detected when *TLR2* rs3804099 and *TLR4* rs11536889 were combined. Periodontopathogenic bacteria were not associated with OC among individuals with the *TLR2* rs3804099 CT or CC + *TLR4* rs11536889 GG genotype combination (OR = 1.09, 95% CI: 0.93–1.26), while the OSCC risk associated with periodontopathogenic bacteria was significantly increased (OR = 1.46, 95% CI: 1.23–1.75) for all other genotype combinations.

Evaluating the influence of alcohol, betel quid and cigarette consumption on the percentage of periodontopathogenic bacteria

Alcohol drinking and betel quid chewing were associated with a significantly higher percentage of *P. intermedia*, while cigarette smoking was associated with a significantly higher percentage of *F. nucleatum* (Table 4). Individuals who used all three of these substances had the highest percentage of periodontopathogenic bacteria, followed by individuals who used one or two of these three substances, and individuals who did not use any had the lowest percentage of periodontopathogenic bacteria.

Assessing the influence of *ADH1B* and *ALDH2* polymorphisms on the association between alcohol and the percentage of periodontopathogenic bacteria

The efficiency for ethanol metabolism may be influenced by the genetic polymorphisms of *ADH1B* and *ALDH2*. Our analysis showed that *ADH1B* polymorphism did not affect the association between alcohol and the percentage of periodontopathogenic bacteria (Table 5). For *ALDH2* polymorphism, we found that among individuals with the *ALDH2*1/*1* genotype, which is associated with normal ALDH2 activity, alcohol drinking showed no significant association with the percentage of periodontopathogenic bacteria. For individuals with either the *ALDH2*1/*2* or *ALDH2*2/*2* genotype, which are both associated with a deficient ALDH2 activity, alcohol drinking was associated with a significantly increased percentage of *P. intermedia* and of all three periodontopathogenic bacteria combined.

Evaluating the association between oral hygiene and the percentage of periodontopathogenic bacteria

Tooth brushing less than two times per day and lack of regular dental visit were both associated with a significantly higher percentage of *P. tannerae*, while no use of dental floss was associated with a significantly higher percentage of *F. nucleatum* (Table 6). When tooth brushing, use of dental floss and regular dental visit were combined into a dental care score (0, 1 = good and 2, 3 = poor), poor dental care was associated with a significantly higher percentage of *P. tannerae*, *F. nucleatum* and the three periodontopathogenic bacteria combined. Poor dental care was also associated with a higher percentage of *P. intermedia*, but statistical significance was not reached ($P = 0.08$).

Table 3. The association between periodontopathogenic bacteria and OSCC by polymorphisms of *TLR2* and *TLR4*

TLR polymorphisms	OR for the association between every 1% of periodontopathogenic bacteria and OSCC[a] (95% CI)[b]	*P* value for heterogeneity
TLR2 rs3804099		
TT	1.48 (1.19–1.83)	0.19
CT+CC	1.19 (1.02–1.37)	
TLR2 rs3804100		
TT	1.35 (1.13–1.61)	0.55
CT+CC	1.21 (1.03–1.43)	
TLR4 rs11536889		
GG	1.19 (1.03–1.38)	0.14
CG+CC	1.48 (1.17–1.88)	
TLR2 rs3804099/*TLR4* rs11536889		
CT+CC/GG	1.09 (0.93–1.26)	0.07
CT+CC/CG+CC	2.04 (1.07–3.87)	
TT/GG	1.66 (1.16–2.37)	
TT/CG+CC	1.82 (1.03–3.23)	
CT+CC/GG	1.09 (0.93–1.26)	0.01
CT+CC/CG+CC or TT/GG or TT/CG+CC	1.46 (1.23–1.75)	

[a]Periodontopathogenic bacteria = *Prevotella tannerae* + *Fusobacterium nucleatum* + *Prevotella intermedia*.
[b]OR and 95% CI were calculated using unconditional logistic regression, adjusted for age, education, and consumption of alcohol, betel quids and cigarettes.

Evaluating the correlation between periodontopathogenic bacteria and cytokine/chemokine levels in the saliva

We analyzed the correlation between the percentage of periodontopathogenic bacteria and the levels of 15 cytokines/chemokines in the saliva from the control subjects (Supplementary Table 3, available at *Carcinogenesis* Online). Because the saliva collection to measure salivary cytokines/chemokines started later than that for the profiling of oral bacteria, only 61 of the 151 controls had samples for cytokine/chemokine measurements. Our results showed that the percentage of periodontopathogenic bacteria was significantly positively correlated with the level of IL1β in the saliva ($P = 0.0009$ for Pearson correlation and $P < 0.0001$ for Spearman correlation) (Supplementary Figure 2 and Supplementary Table 3, available at *Carcinogenesis* Online). Although Spearman correlation indicated that IL2 was positively correlated with the percentage of periodontopathogenic bacteria ($P = 0.02$), chance finding with IL2 could not be ruled out due to multiple comparisons. There were no significant correlations between the percentage of periodontopathogenic bacteria and the levels of CCL20, IFNγ, IL5, IL6, IL9, IL10, IL12p70, IL13, IL15, IL17A, IL21, IL33 or TNFα.

Discussion

We found that three species of periodontopathogenic bacteria, *P. tannerae*, *F. nucleatum* and *P. intermedia*, were associated with an increased OSCC risk. This association was modified by the genetic polymorphisms of *TLR2* and *TLR4*. The use of alcohol, betel quids and cigarettes and poor oral hygiene were associated with a higher percentage of periodontopathogenic bacteria in the saliva. The percentage of periodontopathogenic bacteria in the saliva was positively correlated with the level of salivary IL1β and possibly IL2.

Table 4. The association between use of alcohol, betel quid, cigarette and oral bacteria among control subjects

Lifestyle factors	N	*Prevotella tannerae* Mean % (SE)	P[a]	*Fusobacterium nucleatum* Mean % (SE)	P[a]	*Prevotella intermedia* Mean % (SE)	P[a]	*Periodontopathogenic bacteria*[b] Mean % (SE)	P[a]
Alcohol									
Ever regular drinker									
No	83	0.46 (0.07)	0.43	0.39 (0.04)	0.21	0.59 (0.09)	0.02	1.44 (0.14)	0.08
Yes	68	0.38 (0.06)		0.49 (0.08)		1.19 (0.27)		2.06 (0.35)	
Betel									
Ever betel quid chewer									
No	103	0.44 (0.06)	0.59	0.39 (0.04)	0.13	0.68 (0.11)	0.04	1.51 (0.16)	0.08
Yes	48	0.38 (0.08)		0.52 (0.08)		1.26 (0.34)		2.16 (0.43)	
Cigarette									
Ever cigarette smoker									
No	47	0.44 (0.10)	0.85	0.31 (0.04)	0.03	0.50 (0.12)	0.06	1.24 (0.20)	0.07
Yes	104	0.42 (0.05)		0.49 (0.05)		1.03 (0.18)		1.93 (0.24)	
Alcohol + betel quid + cigarette									
No use	39	0.49 (0.12)	0.70	0.33 (0.04)	0.26	0.52 (0.14)	0.01	1.34 (0.23)	0.047
Use 1 or 2	79	0.39 (0.06)		0.45 (0.06)		0.74 (0.12)		1.58 (0.18)	
Use all 3	33	0.42 (0.10)		0.51 (0.11)		1.58 (0.48)		2.51 (0.61)	

[a]P values were generated using T-tests or analysis of variance.
[b]Periodontopathogenic bacteria = *Prevotella tannerae* + *Fusobacterium nucleatum* + *Prevotella intermedia*.

Table 5. The association between use of alcohol and oral bacteria among control subjects by *ADH1B* and *ALDH2* genotypes

		Prevotella tannerae		*Fusobacterium nucleatum*		*Prevotella intermedia*		*Periodontopathogenic bacteria*[b]	
Alcohol	*N*	Mean % (SE)	P[a]	Mean % (SE)	P[a]	Mean % (SE)	P[a]	Mean % (SE)	P[a]
ADH1B									
*Fast ADH1B*2/*2*									
Ever regular drinker									
No	44	0.43 (0.09)	0.39	0.39 (0.05)	0.18	0.66 (0.14)	0.08	1.47 (0.21)	0.15
Yes	34	0.32 (0.07)		0.56 (0.13)		1.25 (0.34)		2.13 (0.45)	
*Slow ADH1B*1/*1 + ADH1B*1/*2*									
Ever regular drinker									
No	39	0.49 (0.11)	0.75	0.39 (0.05)	0.83	0.53 (0.12)	0.16	1.40 (0.18)	0.31
Yes	31	0.43 (0.11)		0.41 (0.09)		1.13 (0.45)		1.97 (0.58)	
ALDH2									
*Normal ALDH2*1/*1*									
Ever regular drinker									
No	17	0.51 (0.19)	0.50	0.40 (0.07)	0.72	0.59 (0.16)	0.32	1.50 (0.29)	0.58
Yes	45	0.39 (0.08)		0.46 (0.09)		0.95 (0.22)		1.80 (0.31)	
*Slow ALDH2*1/*2 + Non-functional ALDH2*2/*2*									
Ever regular drinker									
No	66	0.44 (0.07)	0.53	0.38 (0.04)	0.14	0.60 (0.11)	0.02	1.42 (0.16)	0.04
Yes	20	0.35 (0.11)		0.56 (0.17)		1.73 (0.76)		2.64 (0.95)	

[a]P values were generated using T-tests.
[b]Periodontopathogenic bacteria = *Prevotella tannerae* + *Fusobacterium nucleatum* + *Prevotella intermedia*.

Table 6. The association between dental care and periodontopathogenic bacteria among controls

		Prevotella tannerae		*Fusobacterium nucleatum*		*Prevotella intermedia*		*Periodontopathogenic bacteria*[b]	
Cigarette	*N*	Mean % (SE)	P[a]	Mean % (SE)	P[a]	Mean % (SE)	P[a]	Mean % (SE)	P[a]
Tooth brushing									
2 or more per day	104	0.36 (0.05)	0.04	0.41 (0.05)	0.34	0.81 (0.13)	0.52	1.57 (0.18)	0.21
<2 times per day	47	0.57 (0.09)		0.49 (0.07)		0.99 (0.31)		2.04 (0.39)	
Use of dental floss									
Yes	58	0.38 (0.07)	0.53	0.33 (0.04)	0.04	0.63 (0.15)	0.16	1.34 (0.20)	0.09
No	93	0.45 (0.06)		0.50 (0.06)		1.01 (0.19)		1.96 (0.25)	
Regular dental visit									
Yes	52	0.28 (0.04)	0.04	0.40 (0.06)	0.51	0.87 (0.30)	0.99	1.55 (0.37)	0.48
No	99	0.49 (0.07)		0.45 (0.05)		0.86 (0.12)		1.81 (0.18)	
Dental care score[c]									
0 + 1 (good)	67	0.31 (0.06)	0.04	0.34 (0.04)	0.03	0.61 (0.14)	0.08	1.26 (0.18)	0.02
2 + 3 (poor)	84	0.51 (0.07)		0.51 (0.06)		1.07 (0.21)		2.09 (0.28)	

[a]P values were generated using T-tests.
[b]Periodontopathogenic bacteria = *Prevotella tannerae* + *Fusobacterium nucleatum* + *Prevotella intermedia*.
[c]Dental care score = tooth brushing + use of dental floss + regular dental visit, with tooth brushing: ≧2 times per day = 0, <2 times per day = 1; use of dental floss: yes = 0, no = 1 and regular dental visit: yes = 0, no = 1. A dental care score of 0 or 1 was considered good oral hygiene where as a score of 2 or 3 was considered poor oral hygiene.

Our result on the positive association between the three species of periodontopathogenic bacteria, *P. tannerae*, *F. nucleatum* and *P. intermedia*, and OSCC gave support and provided a biological link for the association between poor oral hygiene and OC. Of the three periodontopathogenic bacteria, *F. nucleatum* has gained the most attention with its potential role in the development of cancer, including oral cancer, colorectal cancer and pancreatic cancer (43). *F. nucleatum* may potentially increase the occurrence and enhance the progression of cancer by promoting cell proliferation, increasing cellular migration and invasion, and inducing inflammation (43). Binder Gallimidi *et al.* showed that mice co-infected with *F. nucleatum* and *P. gingivalis* generated tongue tumors that were significantly larger and more invasive (44). Furthermore, co-incubation with either *F. nucleatum* or *P. gingivalis* alone or a mixture of both promoted the growth of human OSCC cells (44). This was achieved through the interaction between *F. nucleatum* and/or *P. gingivalis* and TLR2 expressed on the OSCC cells, which triggered the production

of IL6 and the activation of STAT3, resulting in the induction of downstream effectors to promote the proliferation and invasion of OSCC cells (44). Data are lacking on the carcinogenicity of *P. tannerae* and *P. intermedia*. More investigations are needed to decipher the biological mechanisms underlying the association between OSCC and periodontopathogenic bacteria.

Previous studies have reported inconsistent findings regarding the taxa of bacteria associated with oral cancer (45). The sources of heterogeneity may include: (i) different methods for analyzing bacteria (culture versus real-time PCR versus 16S rRNA metagenomics); (ii) different specimen types (saliva versus tissue) and (iii) different types of controls (specimens from healthy controls versus non-cancerous tissues from oral cancer patients) (45). Furthermore, studies with different ethnic groups and different geographic locations may provide additional sources of variations for the composition and diversity of human microbiome (46). Our results did not concur with those of the two studies that employed a similar study design as ours (47,48). However, these two studies had a small sample size [Pushalkar *et al*. included only 3 OSCC cases and 2 controls (47) and Guerrero-Preston *et al*. included 11 oropharyngeal cancer and 6 OSCC cases and 25 controls (48)] and lacked a validation group such that random variations and chance findings of their results could not be ruled out. More studies with a larger sample size are needed to investigate the association between oral microbiome and OSCC. In addition, race/ethnicity- and geography-specific references of microbiome composition need to be established.

Our results showed an interaction between periodontopathogenic bacteria and SNPs of *TLR2* and *TLR4* to influence OSCC risk, with individuals carrying the genotype combination of *TLR2* rs3804099 CT or CC and *TLR4* rs11536889 GG having a weaker association between periodontopathogenic bacteria and OSCC. Rs11536889 is a SNP located in the 3′-untranslated region of *TLR4*. The peripheral blood monocytes of individuals with the rs11536889 CC genotype expressed higher levels of TLR4 protein than those with the GC or GG genotype; however, the mRNA expression levels did not differ by the genotypes, suggesting that rs11536889 regulates the translation and not the transcription of TLR4 (49). Further evidence suggested that the G allele created a binding site for two micro RNAs, hsa-miR-1236 and has-miR-642a, that down-regulated the level of TLR4 (49). Fukusaki *et al*. showed that the CC genotype of rs11536889 was positively associated with moderate and chronic periodontitis (50). This suggested that individuals with the rs11536889 GG genotype, with a lower level of TLR4, may develop a more contained inflammation in response to periodontopathogenic bacteria infection and thus explain the weaker association between the periodontopathogenic bacteria and OSCC among these individuals. *TLR2* rs3804099 (Asn199Asn) is a synonymous SNP with unknown function. A synonymous SNP may influence the processing of mRNA by affecting the splice sites or the transcription factor binding site, alter the structure of mRNA and affect the translational speed for generating protein (51). Despite the unknown function, the CT and CC genotypes of rs3804099 were associated with a decreased cancer risk among Asians (52). This is consistent with our finding that the association between periodontopathogenic bacteria and OSCC risk was weaker for individuals with the rs3804099 CT or CC genotype compared to that for those with the TT genotype.

Our results showed that periodontopathogenic bacteria were positively correlated with the level of salivary IL1β, a pro-inflammatory cytokine. IL1β has been shown to promote the oncogenesis of OSCC by increasing the proliferation of dysplastic oral cells and stimulating the oncogenic cytokines (35). Lipopolysaccharides purified from *F. nucleatum* and *P. intermedia* were shown to induce a significant production of IL1β (53,54). Bui *et al*. showed that infection of gingival epithelial cells with *F. nucleatum* resulted in the translocation of NF-κB into the nucleus to promote the expression of cytokine genes, including *IL1B* (55). Furthermore, *F. nucleatum* was shown to activate NLRP3 inflammasome and caspase-1, resulting in the secretion of IL1β (55). Together, these suggested that periodontopathogenic bacteria may promote the oncogenesis of OSCC by inducing inflammation, particularly through the effect of IL1β. Our result also indicated that the level of salivary IL2 might be positively correlated with the percentage of periodontopathogenic bacteria. Due to multiple testing with 15 cytokines, a weak *P* value of 0.02 could not rule out the chance finding with IL2; however, there may be a biological plausibility for this correlation. Infection with microbes can effectively stimulate the dentritic cells to produce IL2 (56) and this may explain the positive correlation between the percentage of periodontopathogenic bacteria and the level of salivary IL2 in our study.

Our results showed a positive association between the percentage of periodontopathogenic bacteria in the saliva and the use of alcohol, betel quids and cigarettes. In addition, to our knowledge, we are the first to show that *ALDH2* polymorphism may modify the association between alcohol use and periodontopathogenic bacteria, with the association being stronger among individuals carrying the genotypes associated with a lower ALDH2 activity (i.e. slower ethanol metabolism). This gave further support to the influence of alcohol on the percentage of periodontopathogenic bacteria. Previous studies also reported that alcohol drinking and cigarette smoking may affect the composition of oral bacteria. Lages *et al*. observed that alcohol dependence was associated with higher levels of periodontal pathogens, including *P. intermedia*, *F. nucleatum*, and *E corrodens*, in the subgingival samples (57). Moon *et al*. compared the subgingival samples from smoking and non-smoking chronic periodontitis patients and found that smoking was associated with a significantly higher abundance of seven bacterial species, including *F. nucleatum* (58). Mason *et al*. evaluated the subgingival microbiome of 200 individuals with no periodontal diseases and observed that the subgingival microbiome of smokers had a lower abundance of commensal bacteria and was enriched with systemic and periodontal pathogens, including *F. nucleatum* (59). In a study with 1204 adults, Wu *et al*. showed that smoking was associated with depletion of bacteria genera that are associated with carbohydrate and energy metabolism and xenobiotic metabolism (60). Ling *et al*. reported that betel quid use was associated with a higher probability of subgingival infection with periodontal pathogens (61). Overall, the use of alcohol, betel quids and cigarettes may potentially lead to dysbiosis of oral microbiome and increase OSCC risk.

This study has several limitations. First, due to the case–control study design, saliva samples were collected from the OSCC patients before treatment. Therefore, it may be difficult to determine the temporal relationship of our findings. Second, we used saliva to analyze the oral bacterial profiles, which may not be entirely representative due to the many niches of the oral cavity. However, bacterial compositions from the different niches of oral cavity are correlated. He *et al*. compared the bacterial loads of four periodontopathogenic bacteria, including *F. nucleatum* and *P. intermedia*, in the saliva and supragingival and subgingival plaque samples of 84 subjects (60 with chronic periodontitis and 24 with no periodontal disease). They observed that the bacterial loads of the periodontopathogenic bacteria in the saliva

were significantly correlated with those of the supragingival and subgingival plaque samples (Spearman's rank correlation coefficients ranged from 0.26 to 0.33 for *F. nucleatum* and 0.58 to 0.63 for *P. intermedia*) (62). Haririan *et al*. also concluded that saliva sample may be a reasonable alternative to subgingival plaque sample for analyzing bacteria associated with aggressive and chronic periodontitis (63). Finally, our study included only Taiwanese of Han ethnicity and thus the results may not be generalized to other ethnic populations. More investigations with other ethnic populations are needed to determine the similarities and differences.

This study has several strengths. First, our saliva samples were collected specifically to study the profiles of oral bacteria and not as secondary samples collected for other study purposes. We collected the saliva sample using Omnigene-ORAL (OM505) (DNA Genotek Inc), which stabilizes microbial DNA at the time of saliva collection and ensures that the DNA composition is unchanged at room temperature for up to 3 weeks. This minimized the inaccuracy of the results due to continued bacterial growth after sample collection. Second, we accounted for multiple testing in our analysis and had an independent group of study subjects for validation to minimize false positive results. Finally, we not only showed the significant difference in the percentage of periodontopathogenic bacteria between OSCC cases and controls, we also provided other supporting data, including: (i) a significant gene–environment interaction (between periodontopathogenic bacteria and SNPs of *TLR2* and *TLR4*) on OSCC risk; (ii) the positive association between the percentage of periodontopathogenic bacteria and the use of alcohol, betel quids and cigarettes and (iii) the positive correlation between the percentage of periodontopathogenic bacteria and the level of the pro-inflammatory cytokine IL1β in the saliva. These data together corroborated the role of periodontopathogenic bacteria in the development of OSCC.

Overall, our results indicated an increased OSCC risk associated with a higher percentage of oral periodontopathogenic bacteria, including *P. tannerae*, *F. nucleatum* and *P. intermedia*. This association may be influenced by lifestyle and genetic factors. Our results provided biological support for the association between poor oral hygiene and OSCC risk. This suggested that improving oral hygiene may reduce OSCC risk and should be part of a public health campaign to prevent the occurrence of OSCC.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

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