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FULL LENGTH ARTICLE

Salivary microbiota may predict the presence of esophageal squamous cell carcinoma

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Abstract The aim is to explore the predictive value of salivary bacteria for the presence of esophageal squamous cell carcinoma (ESCC). Saliva samples were obtained from 178 patients with ESCC and 101 healthy controls, and allocated to screening and verification cohorts,

Abbreviations: ESCC, esophageal squamous cell carcinoma; PCR, polymerase chain reaction; HTS, high-throughput sequencing; OTUs, Operational Taxonomic Units; Q-PCR, quantitative PCR; ROC, receiver operating characteristic; ECA, esophageal carcinoma; KW, Kruskal-Wallis; LDA, linear discriminant analysis.

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(ESCC);
Predictive value;
Quantitative polymerase chain reaction (Q-PCR);
Receiver operating characteristic (ROC) curves;
Salivary bacteria

respectively. In the screening phase, after saliva DNA was extracted, 16S rRNA V4 regions of salivary bacteria were amplified by polymerase chain reaction (PCR) with high-throughput sequencing. Highly expressed target bacteria were screened by Operational Taxonomic Units clustering, species annotation and microbial diversity assessment. In the verification phase, the expression levels of target bacteria identified in the screening phase were verified by absolute quantitative PCR (Q-PCR). Receiver operating characteristic (ROC) curves were plotted to investigate the predictive value of target salivary bacteria. LEfSe analysis revealed higher proportions of Fusobacterium, Streptococcus and Porphyromonas, and Q-PCR assay showed significantly higher numbers of *Streptococcus salivarius*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* in patients with ESCC, when compared with healthy controls (all $P < 0.05$). The areas under the ROC curves for *Streptococcus salivarius*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and the combination of the three bacteria for predicting patients with ESCC were 69%, 56.5%, 61.8% and 76.4%, respectively. The sensitivities corresponding to cutoff value were 69.3%, 22.7%, 35.2% and 86.4%, respectively, and the matched specificity were 78.4%, 96.1%, 90.2% and 58.8%, respectively. These highly expressed *Streptococcus salivarius*, *Fusobacterium nucleatum* and *Porphyromonas gingivalis* in the saliva, alone or in combination, indicate their predictive value for ESCC.

Introduction

Esophageal carcinoma (ECA), especially esophageal squamous cell carcinoma (ESCC), is a clinically serious disease with a high incidence in China; Chinese patients with ECA account for about 50% of global cases, and approximately 90% of global ESCC cases are in China.[1] According to Global Cancer Statistics 2018, new cases and deaths of ECA are estimated to be about 572,000 and 509,000, respectively, making ECA the seventh most common cancers and sixth most common causes of death worldwide.[2] The incidence of ECA often shows great variation, and is closely related to geography, ethnicity, and living habits.[3] ECA cases exhibit obvious geographical distribution differences in China, with the incidence and mortality gradually increasing from South to East and from Northeast to Central China.[4]

Recently, the association between bacteria and tumorigenesis has been increasingly investigated. Several epidemiological studies from countries with a high incidence of ECA, such as India, Iran, and China, have indicated a potential association between poor oral hygiene and ESCC.[5–8] The dysbiosis of oral microbiota or the increase of pathogenic bacteria is often considered to be a common factor of poor oral hygiene.[9] Moreover, studies have shown that *Porphyromonas gingivalis* infection is associated with oral squamous cell carcinoma.[10] Since oral bacteria can be swallowed with saliva, the bacteria settled in the esophagus are thought to be more derived from the mouth. Previously, we have observed that the levels of *Porphyromonas gingivalis* and *Fusobacterium nucleatum* are elevated in the tissues of ESCC, and *P. gingivalis* promotes proliferation of ESCC cells by activating NF-κB factor.[11] Based on the above observations, we hypothesize the colonization of oral bacteria in the esophagus is associated with the occurrence of ESCC. Therefore, the aim of the present study is to investigate the association between oral microbiota and ESCC, and to explore the predictive value of salivary bacteria for the presence of ESCC.

Patients and methods

Subject selection

All patients with histologically diagnosed ESCC after upper endoscopy prior to any therapeutic procedures, such as surgery or chemoradiotherapy from January 2018 to December 2019 at Guangdong General Hospital were included in the study. Subjects with normal results for chest X-rays, gastroscopy, abdominal ultrasound, blood test and fecal occult-blood test, and digital rectal examinations at their annual physical check-up during the period of time were recruited as healthy controls. Patients and healthy subjects were randomly divided into screening and verification cohorts. Subjects with the following conditions were excluded: a) with concomitant other malignancies, or a history of operation, chemotherapy or radiotherapy for other malignancies; b) with a history of organic/systemic diseases, oral, archenteric, or hepatitis B virus/hepatitis C virus infection; c) with a history of severe diarrhea or administration of drugs that affect oral/salivary flora such as antibiotics, proton pump inhibitors, hormones, intestinal probiotics, etc. within 4 weeks prior to saliva collection; and d) pregnancy or lactation.

The protocol of this study was approved by the Institutional Review Board and Ethics Committee of Guangdong General Hospital (Guangdong Academy of Medical Sciences). Written informed consent was obtained from all the individuals.

Saliva collection

All subjects were asked to refrain from eating, cigarette smoking, alcohol intake to keep oral hygiene for at least 2 h before saliva collection, which was set at 8:00—11:00 am. Up to 5 mL of saliva from each individual was collected into

a 50-mL centrifuge tube after gargling with 10 mL normal saline three times. The collected saliva samples were transported into a refrigerator within 30 min, and stored at −80 °C for subsequent use. All the procedures above were required to be completed within 2 h.

DNA extraction and storage

Saliva DNA was extracted by using UltraClean® Microbial DNA Isolation Kit purchased from QIAGEN in Germany. The DNA was subsequently stored at −20 °C in a refrigerator before utility. The purified DNA met the following quality criteria: the total mass ≥150 ng, DNA concentration ≥5 ng/μL, exhibiting obvious main band, free of degradation, and without contamination of DNA or protein.

Screening phase

16s RNA sequencing and data processing

Patients and healthy subjects allocated to the screening cohort were included in the screening phase.

Distinct V4 regions of 16S rRNA gene were amplified by a specific primer with a 12 bp barcode. Polymerase chain reaction (PCR) amplification with high-throughput sequencing was performed by thermocycling with 5 min initialization at 94 °C, 30 cycles (30 s) denaturation at 94 °C, 30 s annealing at 52 °C, and 30 s extension at 72 °C, followed by 10 min final elongation at 72 °C. PCR products were detected by 1% agarose gel electrophoresis (Guangzhou HaoMa Biotechnology Co., Ltd, Guangzhou, China) for the determination of fragment lengths and concentrations, and by GeneTools Analysis Software (Version4.03.05.0, SynGene) for comparative analysis of the product concentrations. The isodensity proportional mixed PCR products were purified with EZNA Gel Extraction Kit (Omega, USA), and eluted by TE buffer (REGAL, Shanghai, China) to recover the target DNA fragments. The DNA library was established following the standard procedures specified for NEBNextUltra™ DNA Library Prep Kit Illumina® (New England Biolabs, MA, USA). The DNA amplicon library was established by Illumina Hiseq2500 platform for PE250 sequencing, by Trimmomatic Software (V0.33, USADELLAB.org) for filtering Paired-end raw Reads, by Software FLASH (Fast Length Adjustment of SHort reads, V1.2.11,https://ccb.jhu.edu/software/FLASH/) for merging each PE Reads, and by Mothur Software (V1.35.1, http://www.mothur.org) for Raw Tags sequence quality filtering. Finally, all the clean tags (the effective sequences) were obtained.

Bioinformatics analysis

All clean tags were clustered into operational taxonomic units (OTUs) by using UPARSE Software (Version 10, http://www.drive5.com/usearch/) with a cutoff of 97% for identity. Those representative sequences of OTUs read with the highest frequencies were assigned at different taxonomic levels to annotate species, followed by a linear discriminant analysis effect size (LEfSe) tool with QIIME-based wrapper of the Ribosomal Database Project (RDP) classifier. LEfSe analysis was used to identify microbiomes of biomarker properties at multiple levels from salivary samples, with quantitative classification according to statistical significance, and to visualize the results using taxonomic bar charts and cladograms.

Verification phase

Patients and healthy subjects allocated to the verification cohort were included in the verification phase.

The expression levels of target bacteria were verified by quantitative PCR (Q-PCR). After specific primer sequences were designed according to selected target bacteria, the concentration of the positive recombinant plasmid was determined by an ultraviolet spectrophotometer. Then, the plasmid was diluted 10 times, and samples with concentrations of 10-2, 10-3, 10-4, 10-5, 10-6 and 10-7 were used to draw the standard curve. The reaction system was added with sterile water to 20 μL, involving SYBR Mix 10 μL, upstream and downstream primers 0.6 μL each, DNA template 1 μL. Reaction conditions involved one cycle of 30 s at 95 °C, one cycle of 5 s at 95 °C, and 40 cycles of 30 s at 60 °C, followed by DNA dilution of each sample at a concentration of 100 ng/μL used to detect DNA content of the sample. After each sample test was repeated 3 times and the average value was taken, the target bacterial expression, represented by the copy number, in the sample was calculated by the standard curve.

Receiver operating characteristic (ROC) curves were plotted based on detected saliva target bacteria expression in patients and healthy subjects, combined with indicators such as gender, age, cigarette smoking, alcohol intake and preference for hot food. The greater the AUC value the greater the predictive value of target bacteria for ESCC.

Statistical analysis

SPSS 22.0 Software (IBM, version 22.0) was used for statistical analysis, and $P < 0.05$ was considered statistically significant. Mann-Whitney *U* test was performed to compare the differences in the relative abundance of bacteria, and independent sample *t*-test was used to detect the differences in age and bacterial copy quantity, and Chi-square test was used to detect the differences in sex, cigarette smoking, alcohol intake, hot food preference between ESCC patients and healthy subjects. In terms of LEfSe analysis, the two-tailed non-parametric factorial Kruskal-Wallis sum-rank test was first used to identify the bacterial species with significant differences in abundance between ESCC patients and healthy subjects, and then Wilcoxon rank-sum test was used to compare the differences between the two groups. Linear discriminant analysis (LDA) was used to achieve dimensionality reduction and assess the impact (LDA Score, with a filter value of 2) of species with significant differences. Finally, SPSS 22.0 Software was used to draw the ROC curve.

Results

Basic demographic characteristics of the enrolled subjects

A total of 178 patients with ESCC (90 cases in screening cohort, and 88 cases in verification cohort), and 101 healthy

subjects (50 cases in screening cohort, and 51 cases in verification cohort) were eligible for the study. The average age of screening and verification sets in patients with ESCC was 61.7 and 61.4 years, respectively, and that of screening set in healthy subjects was 43.9 and 44.4 years, respectively. The demographic characteristics of the enrolled subjects are presented in Table S1.

Target bacteria identified in the screening phase

All clean tags (effective tags) were clustered by OTUs according to 97% sequence similarity, and the community composition of each sample was analyzed at phylum and genus levels.

At the phylum level, the dominant bacteria, with a relative abundance of $\geq$1%, were Bacteroidetes, Proteobacteria, Firmicutes, Fusobacteria, Actinobacteria, Spirochaetes, Epsilonbacteraeota, and Patescibacteria in patients with ESCC and healthy subjects (Fig. 1A). Although there was little difference in species composition, the abundance ratio varied greatly between the two groups. Compared with healthy subjects, patients with ESCC showed an increased abundance of Firmicutes, Fusobacteria, Actinobacteria, Spirochaetes and Epsilonbacteraeota, and decreased abundance of Bacteroidetes, Proteobacteria and Patescibacteria (Fig. 1A).

At the genus level, patients with ESCC showed mainly composed bacteria of Neisseria, Fusobacterium, Streptococcus, Hemophilus and Porphyromonas, and healthy subjects showed mainly composed species of Neisseria, Prevotella_7, Hemophilus, Fusobacterium and Alloprevotella. There were higher proportions of Fusobacterium, Streptococcus and Porphyromonas in patients with ESCC (9.3%, 7.8% and 6.9%, respectively) than in healthy subjects (8.1%, 4.9% and 3.0%, respectively). The histogram of the relative abundance of dominant bacteria $\geq$1% in the two groups is shown in Figure 1B.

The histogram of LDA value distribution, which was obtained by LEfSe analysis and illustrates the species with significant enrichment in patients with ESCC and healthy subjects, is shown in Figure 2. Briefly, there were 28 bacteria with significant differences between the two groups; 14 including Streptococcus, Fusobacterium, Porphyromonas, Firmicutes and Bacillus, etc. were more abundant in patients with ESCC, and 14 including Prevotellaceae, Bacteroidales, Bacteroidetes, Bacteroidia and Proteobacteria, etc. were more abundant in healthy subjects (all $P < 0.05$). The abundance comparison diagram of biomarkers (i.e. salivary bacteria of biomarker properties) in each sample of the two groups is shown in Figure 3. Compared with healthy subjects, the patients with ESCC had a dramatically increased abundance of Streptococcus, Fusobacterium and Porphyromonas (all $P < 0.05$).

Target bacteria confirmed in the verification phase

The design sequences of specific primers for *Streptococcus salivarius, Fusobacterium nucleatum* and *Porphyromonas gingivalis* are shown in Table S2.

The correlation coefficients of these standard curves of *S. salivarius, F. nucleatum* and *P. gingivalis* were 0.995, 0.990 and 0.998, respectively. The amplification curves of *S. salivarius, F. nucleatum and P. gingivalis* showed well repeatability, and the single peak characteristics of standard melting curves of the three strains indicated the primer well specificity.

The average copies detected from salivary *S. salivarius, F. nucleatum* and *P. gingivalis* in patients with ESCC and healthy subjects are shown in Table 1. According to the standard curves, the numbers of *S. salivarius, F. nucleatum* and *P. gingivalis* in saliva samples in patients with ESCC

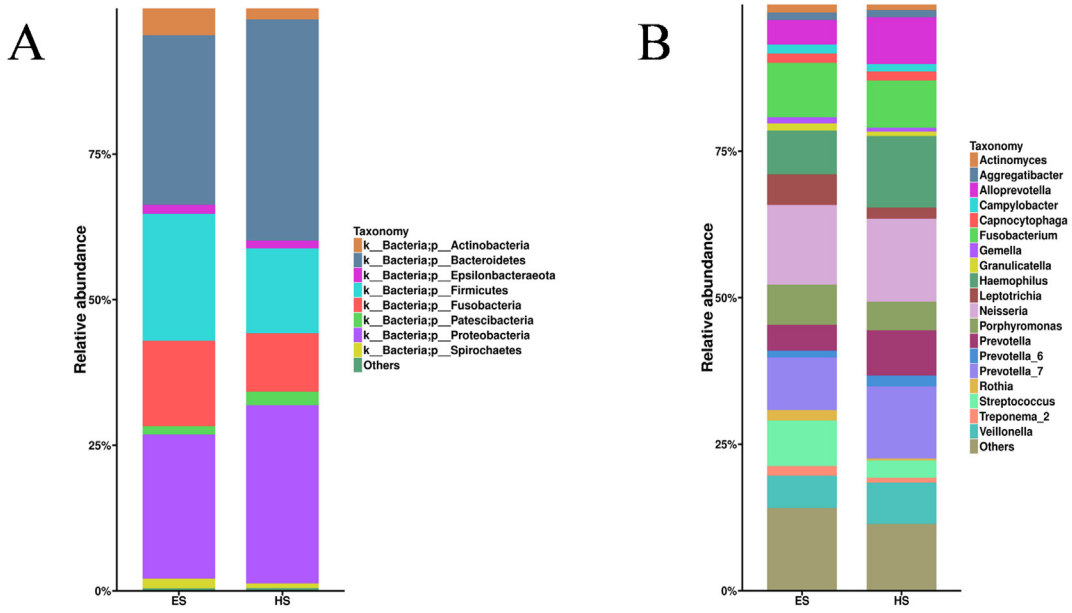

Figure 1 The relative abundance of dominant salivary bacteria at the phylum **(A)** and genus **(B)** levels in patients with esophageal squamous cell carcinoma (ES) and healthy subjects (HS).

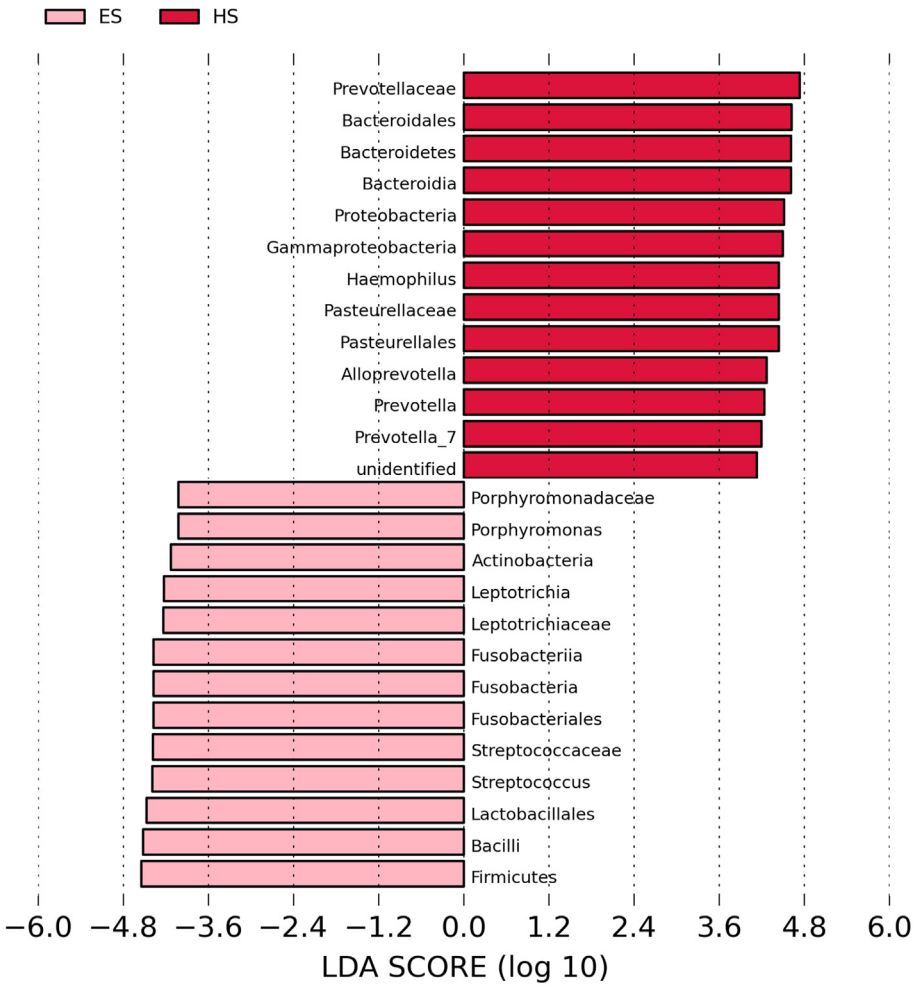

Figure 2 Histogram of the Linear discriminant analysis (LDA) scores calculated for differentially abundant bacteria at the genus level between patients with esophageal squamous cell carcinoma (ES) and healthy subjects (HS).

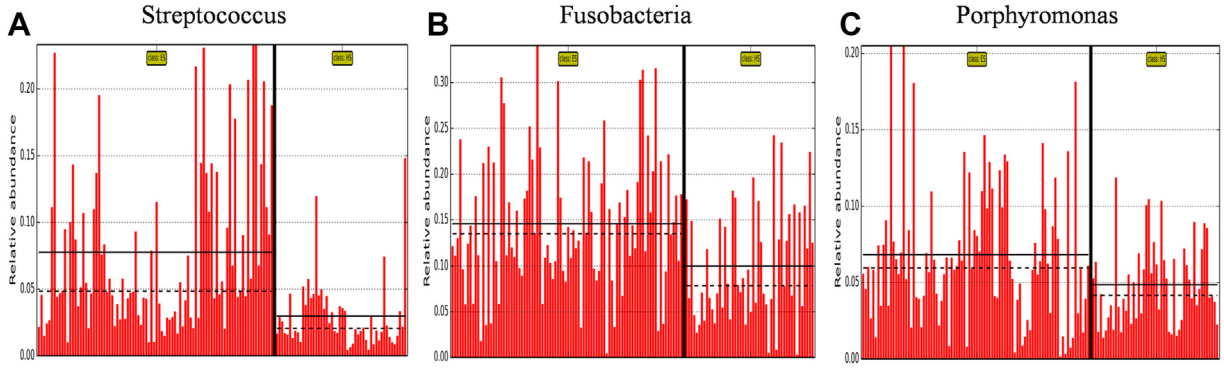

Figure 3 Biomarker (i.e. salivary bacteria of biomarker properties) images of Streptococcus **(A)**, Fusobacteria **(B)** and Porphyromonas **(C)** at the genus level in patients with esophageal squamous cell carcinoma (ES) and healthy subjects (HS).

were 9.00×10^5, 3.10×10^5 and 2.38×10^7, respectively, which were all significantly higher than that (4.85×10^4, 5.32×10^4 and 6.24×10^6, respectively) in healthy subjects (all $P < 0.05$).

The ROC curves were plotted to verify the expression levels (i.e. copy numbers) of salivary *S. salivarius*, *F. nucleatum* and *P. gingivalis* in the patients with ESCC and healthy subjects. The ROC curves of *S. salivarius*, *F. nucleatum*, *P. gingivalis* and the combination of the three bacteria for patients with ESCC are shown in Figure 4A. The AUCs of ROC curves were 69.0%, 56.5%, 61.8% and 76.4%, respectively. The sensitivities and

Table 1 Average copies of *Streptococcus salivarius, Fusobacterium nucleatum and Porphyromonas gingivalis* detected from verification phase in the saliva of patients with esophageal squamous cell carcinoma and healthy subjects.

Fungus name	ES group	HS group	*P* value
S.salivarius (mean ± SD)	$9.00 \times 10^5 \pm 4.44 \times 10^6$	$4.85 \times 10^4 \pm 9.05 \times 10^4$	0.015
F.nucleatum (mean ± SD)	$3.10 \times 10^5 \pm 1.27 \times 10^6$	$5.32 \times 10^4 \pm 1.11 \times 10^5$	0.018
P.gingivalis (mean ± SD)	$2.38 \times 10^7 \pm 8.85 \times 10^7$	$6.24 \times 10^6 \pm 7.98 \times 10^6$	0.031

ES, patients with esophageal squamous cell carcinoma; HS, healthy subjects; SD, standard deviation.

specificities corresponding to the cutoff values were 69.3%, 22.7%, 35.2% and 86.4%, and 78.4%, 96.1%, 90.2% and 58.8%, respectively.

When these factors (patient age, gender, cigarette smoking, alcohol intake, and hot food) were incorporated in the analysis, the AUCs of ROC curves increased to 92.9%, 92.4%, 93.5%, and 93.9%, respectively, for *S. salivarius*, *F. nucleatum*, *P. gingivalis* and the combination of the three bacteria. The sensitivities and specificities corresponding to the cutoff values were 85.2%, 78.4%, 86.4% and 83.0%, and 86.3%, 90.2%, 88.2% and 90.2%, respectively (Fig. 4B).

Discussion

The present study demonstrated significantly increased *S. salivarius, F. nucleatum* and *P. gingivalis* in the saliva of patients with ESCC, when compared with healthy subjects, indicating that these salivary bacteria are the major representative microbiota in oral cavity or salivary liquid of patients with ESCC. These findings also suggest that *S. salivarius, F. nucleatum* and *P. gingivalis* in the saliva are associated with the development of ESCC, and may predict the presence of ESCC.

The pathogenesis of ESCC has not been fully elucidated. Moreover, accurate screening of patients with ESCC remains challenging as most patients are diagnosed in the advanced stage and lose the opportunity for curable therapy.[1] Therefore, a simple and feasible screening method is urgently needed for the early diagnosis of ESCC. Saliva is the nutritional basis for the survival of oral bacteria, which can migrate into the esophagus with the swallowing of saliva, and thus, the flora of the esophageal mucosa is theoretically considered to be derived from the oral cavity. Furthermore, the present study, along with our previous study,[11] clearly demonstrated that salivary bacteria, particularly the proportions of salivary Streptococcus, Fusobacterium and Porphyromonas are significantly increased in patients with ESCC. Additionally, the present study demonstrated that older age, male gender, smoking, alcohol intake and hot food preference were associated with ESCC, which confirms previous findings that these are the risk factors for the development and poor prognosis of ESCC.[12–14]

It has been reported that genes, proteins and small-molecules produced by oral/salivary bacteria are closely related to the occurrence of various cancers. Chemical carcinogens and/or inflammatory factors produced by oral/salivary bacteria, can form a complex and stable bacterial community and play an important pathogenic role in the development and progression of various local and systemic diseases.[15–25] These oral/salivary bacteria-related diseases include, but are not limited to, oral squamous cell carcinoma,[10,26] ESCC,[11] inflammatory bowel disease,[27] pancreatic cancer,[28] celiac disease,[29] and cardiovascular disease.[30–32] In the present study, we further confirmed that salivary bacteria are associated with the development of ESCC, at the genus and species levels.

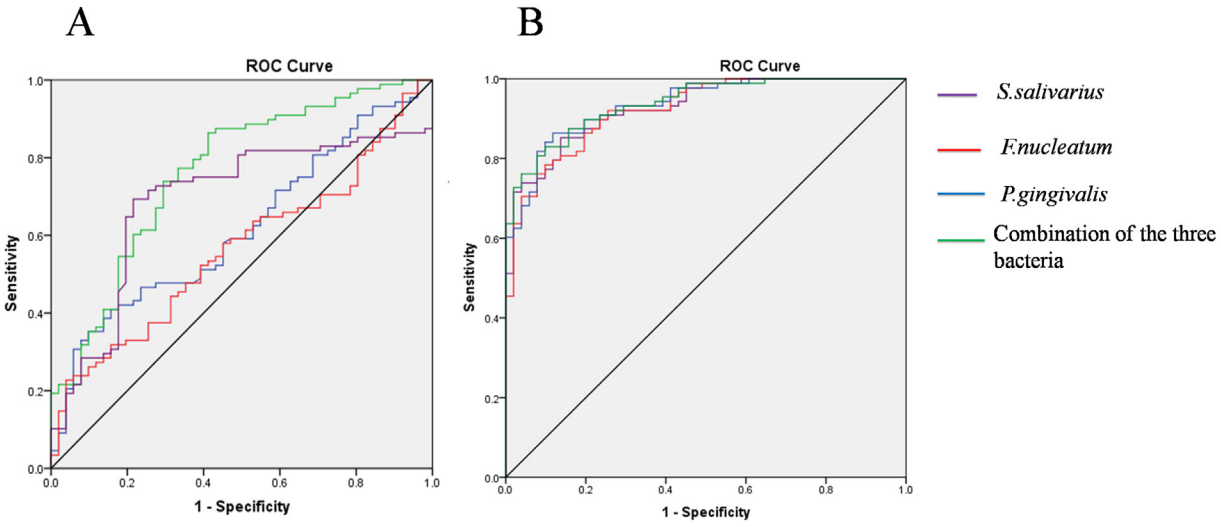

Figure 4 Receiver operating characteristic (ROC) curves of *Streptococcus salivarius, Fusobacterium nucleatum,* and *Porphyromonas gingivalis* and the combination of the three bacteria alone **(A)** and incorporated with age, gender, cigarette smoking, alcohol intake, and hot food preference **(B)** for predicting esophageal squamous cell carcinoma.

The genus Streptococcus is a bacterium that can locate on the oral mucosal surface, as well as the intestinal mucosa. It has been found that Streptococcus increases in oral squamous cell carcinoma tissues.[33] Previous studies have found that Streptococcus and Streptococcus-reactive cytotoxic T cells are positively correlated with relapse-free survival after oral squamous cell carcinoma resection.[26,33] However, the relationship between Streptococcus and ESCC has not been fully elucidated. Fusobacterium is involved in the occurrence of inflammatory diseases and may promote tumorigenicity by causing chronic inflammation.[34,35] Fusobacterium is associated with oral squamous cell carcinoma, and Porphyromonas is the main pathogen of periodontitis, and closely related to the development of oral squamous cell carcinoma.[10]

F. nucleatum often colonizes in the mucosa of the oral cavity, vagina and gastrointestinal tract, and is reportedly to be a pathogen of periodontitis, chorioamnionitis and inflammatory bowel disease, and associated with colonic cancer and adenomas.[36–38] Moreover, Yamamura et al[39] demonstrated that the abundance of *F. nucleatum*in ESCC tissues was significantly higher than that in the normal esophageal mucosal tissues, and promoted tumor aggressive behavior by activating chemokines. *P. gingivalis* belongs to genus Porphyromonas, and is thought to be one of the pathogenic factors of periodontitis. Gao et al[40] showed a significantly higher detection rate of *P. gingivalis* in ESCC tissues than in the adjacent normal tissues, and revealed positive associations of *P. gingivalis* with cell differentiation and metastasis of ESCC. Peters et al[41] using 16S rRNA gene sequencing technology, observed abnormally increased oral *P. gingivalis* in the mouth wash samples, which was associated with lymph node metastasis and poor survival of patients with ESCC. Recent studies have shown that *P. gingivalis*[42] and *F. nucleatum*[43,44] promote oral squamous cell carcinoma and colorectal cancer by activating inflammatory NF-κB pathways. Similarly, *P. gingivalis* is found to promote proliferation and activity of ESCC cells by regulating NF-κB pathway *in vitro*.[11]

It has been reported that immunohistochemistry of serum thyroid transcription factor-1, pheochromoprotein A, CK5/6, CK18 and P63 yields detection rates of 53.7%, 72.8%, 17.7%, 94.9% and 100.0%, respectively, for ESCC.[45] Recent studies have found an increased serum expression in exosome microRNA (i.e. miRNA-21) in ESCC.[46] Serum POU3F3 may be a potential biomarker for ESCC diagnosis owing to its diagnostic sensitivity of 72.8% and specificity of 89.4%.[47] As an indicator of monitoring health and disease, salivary bacteria have diagnostic advantages in screening many cancers.[48] Using microarray technology, our previous study found that the expression of miRNA-10b, miRNA-144, and miRNA-451 in the whole saliva and miRNA-10b, miRNA-144, miRNA-21, and miRNA-451 in salivary supernatant were significantly up-regulated in patients with ESCC.[49] The present study showed significantly increased expression of *S. salivarius, F. nucleatum* and *P. gingivalis* in the saliva, indicating their predictive value for the presence of ESCC. Therefore, monitoring these salivary bacteria is expected to offer a novel, simple, noninvasive and promising screening approach for patients with ESCC.

Limitations

A few limitations exist in the present study. First, only salivary flora at genus level was sequenced by 16S rDNA sequencing in screening phase. Second, only representative species in each genus (i.e. *S. salivarius, F. nucleatum and P. gingivalis*) were selected for Q-PCR quantification and ROC curve plotting, according to our previous studies.[11] Therefore, this study is still at exploratory stage, and the conclusions need to be verified by future large-scale sample studies at molecular and genetic levels. Third, all recruited patients in the present study were confirmed with ESCC, so whether these 3 bacteria could be used for early diagnosis remains uncertain. Thus, an independent validation study is needed to confirm whether the changes in abundances of these 3 bacteria already occur in patients with precancerous lesions. Fourth, the present study mainly focused on the association between oral microbiota and ESCC and the predictive value of salivary bacteria for the presence of ESCC, but did not investigate the causes of changes in saliva microorganisms. It has been reported that the composition of salivary microbiota may be greatly affected by clinical, demographic and environmental factors including age, gender, dietary intake, smoking, and lifestyle habits.[50] More extensive investigation is also required to confirm whether the observed microbial alteration is solely attributed to disease rather than other clinical and environmental factors. Fifth, to investigate the association between bacteria in saliva and esophageal cancer, the ideal design is to choose healthy subjects with young ages as the control group as their flora in saliva is closer to the normal status. Therefore, we chose younger people as the controls.

Conclusions

There is a significantly increased expression of *S. salivarius, F. nucleatum* and *P. gingivalis* in the saliva of patients with ESCC. These bacteria, alone or in combination, have a predictive value for the presence of ESCC. The predictive value can be further improved when age, gender, cigarette smoking, alcohol intake and hot food preference are taken into account.

Funding

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Ethical approval

This study was approved by the Ethics Committee of Guangdong General Hospital (Guangdong Academy of Medical Sciences). All procedures performed in the study involving human participants were in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments.

Informed consent

Written informed consent was obtained from each of the patients or their immediate family member.

Data availability statement

The datasets used during the current study are available from the corresponding author upon reasonable request.

Author contributions

Conceived and designed the experiments: Z.Li.
Performed the experiments: J.Wei, R.Li, Y.Lu.
Analyzed the data: J.Wei, R.Li, Y.Lu.
Contributed reagents/materials/analysis tools: Y.Lu, F.Meng, B.Xian, X.Lai, D.Yang.
H.Zhang, X.Lin, Y.Deng, L.Li, X.Ben, G.Qiao, W.Liu.
Wrote the paper: J.Wei, R.Li, Y.Lu, Z.Li.

Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gendis.2021.02.006.

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