Tumor-Associated Microbiota in Esophageal Squamous Cell Carcinoma

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Research

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Abstract

Background: Human intestinal tract microbiome dysbiosis plays an emerging pivotal role in tumorigenesis of gastrointestinal tract cancers. For esophageal squamous cell carcinoma (ESCC), the esophageal microbiota plays a critical role during the pathogenesis. The microbiome of esophageal can impact its host decades before the onset of ESCC, and can interact with the host's physiological situation, which are affected by lifestyle factors, including diet, obesity, alcohol and tobacco use, and oral hygiene. Our objective is to analyze the composition of the ESCC-associated microbiota and characterize its contribution to the development of ESCC. The esophageal microbiota was prospectively investigated in 18 patients with ESCC and 11 patients with physiological normal (PN) esophagus by 16S rRNA gene profiling, using next-generation sequencing.

Results: The microbiota composition in tumor tissues of ESCC patients is significantly different from that of patients with PN tissues. The ESCC microbiota was characterized by reduced microbial diversity, by decreased abundance of *Bacteroidetes*, *Fusobacteria* and *Spirochactes*. Employing these taxa into a microbial dysbiosis index demonstrated that dysbiosis microbiota had good capacity to discriminate between ESCC and PN esophagus. Functional analysis of the microbiota characterized that ESCC microbiota had altered nitrate reductase and nitrite reductase functions when compared with PN group. The observations were confirmed in other validation cohorts.

Conclusions: Detailed analysis of the microbiota of the ESCC patients revealed that tumor exhibit a dysbiotic microbial community when compared with PN groups. We characterized microbial compositional changes, and further identified significant enrichments of *Treponema amylovorum*, *Streptococcus infantis*, *Prevotella nigrescens*, *Porphyromonas endodontalis*, *Veillonella dispar*, *Aggregatibacter segnis*, *Prevotella melaninogenica*, *Prevotella intermedia*, *Prevotella tannerae*, *Prevotella nanceiensis* and *Streptococcus anginosus* in ESCC oncogenic progression.

Trial registration number: This study was registered at the Chinese Clinical Trial Registry (ChiCTR1800018897). <http://www.chictr.org.cn/>.

Background

Esophageal cancer is the ninth most common cancer and the sixth most common cause of cancer death worldwide [1, 2]. The two major subtypes of esophageal carcinoma are adenocarcinoma and esophageal squamous cell carcinoma (ESCC), with the latter accounting for the dominate histopathological type in the Chinese population [3]. These two subtypes are epidemiologically and biologically distinct. Despite reasonable progress in the diagnosis and treatment of ESCC, the prognosis of the patients remains very poor, with a 5-year survival rate of only about 15–25% [2, 4]. The pathogenesis of ESCC has not been well elucidated, which restricts the effective prevention and treatment of this disease. The incidence of esophageal carcinoma remains a health care challenge worldwide. For ESCC, there are very few targeted therapies available and survival rates remains dismal. Therefore, there is an urgent need to characterize

biomarkers/therapy strategy for esophageal carcinoma. Further studies are needed to clarify the pathogenesis of esophageal cancer and to explore new diagnostic and therapeutic possibilities.

The intestinal tract microbiota, containing at least 38 trillion bacteria, is critical for the maintenance of homeostasis and health [5]. Progress in metagenome-wide association studies of fecal samples has characterized some important microbial markers of colorectal cancer (CRC) [6, 7], and the causal effect of bacteria on cancer has been recognized[8]. Also, the microbiome has been discovered to be involved in the initiation and progression of various types of cancer, such as liver cancer [6, 9, 10]. Experimental evidence indicates that the human intestinal microbiome can influence tumor development and progression in the gastrointestinal tract by damaging DNA, activating oncogenic signaling pathways, producing tumor-promoting metabolites, and suppressing the antitumor immune response [6, 7, 11, 12, 13, 14]. Esophagus is an important part of the upper digestive system and more and more attentions are paid to the relationship between microbiome and esophageal cancer. Esophageal cancer is one of the most aggressive malignant cancers. Treatment strategies provided by conventional therapies have limited improvements in clinical outcome. It is then critical to seek innovative clinical strategies for treating this type of cancer. As intestinal tract microbiota plays important roles during tumorigenesis, exploiting microbiota for cancer prevention or treatment may be feasible.

However, only a small number of studies characterized the human esophageal microbiota in health and disease[15]. The major findings in esophageal adenocarcinoma were that lipopolysaccharides, a major structure of the outer membrane in gram-negative bacteria, can upregulate gene expression of proinflammatory cytokines via activation of the Toll-like receptor 4 and NF- κ B pathway and promote the occurrence of Barrett esophagus and adenocarcinoma[16, 17]. Host interactions with microbiota in esophagitis, Barrett's esophagus, esophageal adenocarcinoma and ESCC can be different. Here we focused on the microbiota of ESCC. As for ESCC, the microbiome was less well characterized. Only few studies suggested that the change of microbiota such as *Fusobacterium* were associated with the occurrence of ESCC [18, 19]. The association between the change of esophageal microbiome and ESCC development has not been well elucidated [15, 20]. Therefore, to investigate the relationship between the changes in esophageal mucosal microbiota and the occurrence of ESCC, we conducted a prospective study and performed high-throughput profiling of the esophageal mucosal microbiota in ESCC cases and normal controls. The next-generation sequencing (NGS) of the 16S rRNA gene were used to determine microbiota communities potentially associated with ESCC. We have demonstrated microbial relative abundances at the phylum and genus level for ESCC. We illustrated the impact of ESCC-associated bacterial taxa during the pathogenesis of ESCC.

Methods

Patients

This prospective observational study was conducted according to a protocol approved by the respective Institutional Ethics Committees of the First Affiliated Hospital of Sun Yat-sen University and according to

the Declaration of Helsinki. This GASTO1039 study was registered at <http://www.chictr.org.cn/> (the Chinese Clinical Trial Registry: ChiCTR1800018897). Eighteen patients with esophageal squamous cell carcinoma (ESCC) and eleven volunteers (individuals with physiological normal (PN) esophagus and no evidence of esophageal diseases) from the First Affiliated Hospital of Sun Yat-sen University were included in the discovery cohort between October 2018 to March 2019 (Supplementary table S1 and S2). A validation cohort of an additional 20 ESCC patients and 4 volunteers were enrolled between from April 2019 to December 2019 (Supplementary table S1 and S2). Tissues for analysis came from patients undergoing routine esophagogastroduodenoscopy for the investigation of upper gastrointestinal symptoms or surgical resection. All patients provided written informed consents.

16S rDNA gene sequencing

DNA was extracted from healthy group (PN group) and tumor group (T group) according to the protocol of E.Z.N.A.® Bacterial DNA Kit (Omega Bio-tek, Norcross, GA, USA). The 16s rDNA V4 hypervariable region was amplified using primers 515F 5'-GTGCCAGCMGCCGCGGTAA-3' and 806R 5'-GGACTACHVGGGTWTCTAAT-3'. PCR products were purified with AmpureXP beads to keep the target fragment. At the end 2*250bp paired-end reads were generated by sequencing on the Illumina HiSeq2500 platform. The primers were assessed using PrimerProspector Software Package (see Supplementary figure S1).

Sequencing data analysis

High-quality data were acquired as the in-house procedure from BGI Co., Ltd, China (Shenzhen, China to eliminate low-quality reads, N reads and so on. Then the clean reads were overlapped to obtain tags using FLASH (v1.2.11)[21]. The clean tags which were dereplicated and filtered singletons to cluster into operational taxonomic units (OTUs) at 97% sequence similarity using USEARCH (v9.1.13) in order to obtain OTUs representative sequences and otu abundance in each sample[22]. Every OTU were assigned to the Greengene Database (v201305, <http://greengenes.secondgenome.com/downloads>) at the similarity threshold of 0.5 using RDP Classifier (v2.2) [23, 24]. Alpha diversity analysis was performed by mothur (v1.31.2)[25]. The nonparametric tests were adopted in alpha diversity analysis. Wilcoxon rank sum test was used between two groups, and Kruskal test was used in the comparison of three groups or more than three groups in alpha diversity analysis. Beta diversity analysis were assessed by QIIME (v1.9) [26]. The results of beta diversity were showed by the principle coordinate analysis (PCoA) of weighted and unweighted UniFrac distance. Differences in beta diversity were evaluated by ANOSIM analysis of similarity and Mantel correlation analysis with 999 permutations[27].

Differential taxonomy analysis

The comparisons of genera relative abundance were performed by Metastats (<http://metastats.cbcb.umd.edu/>) and linear discriminant analysis (LDA) effect size (LEfSe) between PN and T groups[28, 29]. The genus with greater than 3 at the base of P-value <0.05 were considered to choose.

Functional metagenome predictions

We predicted the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Clusters of Orthologous Groups (COG) function by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt, v1.0.0) [30], after constructing the close reference of OTU representative sequences from the discovery cohort Qiime software. The accuracy of the predicted metagenome was evaluated by the Nearest Sequenced Taxon Index (NSTI) value. NSTI demonstrates the mean value of phylogenetic distance between all microbiota OTUs and closest sequenced genomes in one sample. The NSTI value is lower, the accuracy of the predicted metagenome is higher. The predicted KEGG and COG function analysis by STAMP software using two-sided test with Welch's t-test corrected with Benjaminin-Hochberg FDR [31]. The LEfSe analysis was based on the relative abundance of predicted KEGG Pathways. The correlation analysis was performed between the relative abundance of predicted KEGG Level 2 Pathways and microbial dysbiosis index (MDI).

Logistic regression and receiver operating characteristic (ROC) analyses

ROC curves were constructed to reflect the discriminatory potential of the microbiota abundance to detect esophageal cancer. ROC curves and P-values were analyzed according to Wilson/Brown method recommended by GraphPad Prism v8.0.2. Good reproducibility is showed by area under the curve (AUC) results. AUC represents the area under the curve, and CI represents the confidence intervals. The AUC value is higher, and the accuracy of ROC curve is better.

Microbial dysbiosis index (MDI)

The MDI was determined as the log transformation of the ratio between the total abundance of genera increased in Tumor groups and the total abundance of genera decreased in Tumor groups. *Acinetobacter* spp., *Blastomonas* spp., *Klebsiella* spp., *Pseudomonas* spp., *Lactococcus* spp., *Thermus* spp., *Anoxybacillus* spp., *Geobacillus* spp. were included as decreased in Tumor groups, and *Campylobacter* spp., *Fusobacterium* spp., *Non-Fusobacterium* *Fusobacteria*, *Parvimonas* spp., *Peptostreptococcus* spp., *Selenomonas* spp., *Streptococcus* spp., *Veillonella* spp., *Prevotella* spp., *Treponema* spp., *Capnocytophaga* spp. were included as increased in Tumor groups.

Results

16S rRNA microbiota profiling in physiological normal esophagus and ESCC

We compared the 16S rRNA gene of the esophageal microbiota between patients with ESCC (T) and patients with physiological normal esophagus (PN) by NGS. After sequencing and quality filtering, more than 1171914 million clean Tags were obtained corresponding to a mean of 310 OTU and clean tags 40410 per sample. The number of clean Tags was not significantly different between PN and T groups (supplementary figure S2A). To determine the number of biologically significant OTUs, we classified the

OTUs of PN and T groups according to the sequences of the Greengenes Named Isolated database. The results indicated that the frequency of bacteria OTUs was major fraction in OTU classification (supplementary figure S2C). Furthermore, Venn diagram displaying the number of common and specific OTUs identified between PN and N groups (supplementary figure S2D).

The profile of esophageal microbiota demonstrates difference in physiological normal esophagus and ESCC

We computed the alpha diversity of microbes of the T and PN using the OUTs, Shannon index, Simpson index and Good's coverage. On average, patients with ESCC had a significantly lower number of OUTs than PN esophagus (Fig. 1A and supplementary figure S2F). However, the Shannon index and Simpson index were not significantly different between the two groups (Supplementary figure S2B and E). To estimate the bacterial diversity, we used Good's coverage estimator to determine the proportion of total bacterial species represented in samples of each group. Statistical analysis of Good's coverage showed that T groups had significantly different numbers of species when compared with PN groups (Fig. 1B) by measuring beta diversity using both unweighted and weighted UniFrac phylogenetic distance matrices. The microbiota composition of patients with T groups was significantly different from that of PN groups (ANOSIM $R = 0.7879$, $P = 0.001$; and ANOSIM $R = 0.6346$, $P = 0.001$, for unweighted and weighted distances, respectively; Fig. 1C and D).

Age is one of the risk factors for ESCC development. Since patients with ESCC were significantly older than patients with PN esophagus in our cohorts (supplementary table S1), we next asked whether the microbial profile was different between the two groups. Overall, age factor was not influenced the microbiota profiles of full sample set (supplementary figure S3A and B). However, compared with age-matched microbiota in patient with ESCC and PN esophagus using unweighted and weighted UniFrac distance matrices, we found that microbiota composition was significant in the two clinical settings (supplementary figure S3C and D). Furthermore, in the age-matched comparisons, the microbial alpha diversity in ESCC patients was dramatic reduced (supplementary figure S3E, $p = 0.001$). Intriguingly, in the cancer progress comparisons, we found that patients with ESCC had significantly decreased microbial diversity than patients with PN and PreT (pre-cancer) (supplementary figure S4C). The microbiota composition of patients with T groups was significantly different from that of PN and PreT groups (ANOSIM $R = 0.53029$, $P = 0.001$; and ANOSIM $R = 0.4792$, $P = 0.001$, for unweighted and weighted distances, respectively; supplementary figure S4F and G). The relative abundance of *Fusobacterium* spp. gradually increased from PN esophagus to ESCC, while the abundance of Proteobacteria was decreased (Supplementary figure S4E). However, there are no statistically significant differences in the microbiota profiles of ESCC patients with gender and tumor stage (supplementary figure S4A, B, D, H and I). Altogether, these results showed that there are significant decreased in microbial diversity and composition in ESCC.

The abundance of *Fusobacterium* spp. affects the microbiota composition of physiological normal esophagus and ESCC

Overall, the esophageal microbiota was dominated by seven phyla: *Fusobacteria* (7.43%), *Actinobacteria* (0.7%), *Bacteroidetes* (28.93%), *Firmicutes* (35.76%), *Proteobacteria* (23.21%), *Spirochetes* (1.57%), and *Thermi* (1.91%). The ESCC microbiota had an over-representation of *Fusobacteria* ($P < 0.001$), *Bacteroidetes* ($P = 0.002$) and *Spirochactes* ($P < 0.001$) and lower abundance of *Proteobacteria* ($P = 0.006$) and *Thermi* ($P = 0.004$; Fig. 2A). In addition, a significant negative correlation was observed between *Fusobacteria spp.* and *Klebsiella. spp.* ($r = -0.822$, $P < 0.001$; Fig. 2B). Accordingly, the microbiota profiles of the two groups could be discriminated by the abundance of *Fusobacterium spp.* (Mantel correlation, $r = 0.4374$, $P = 0.001$; Fig. 2C) and *Klebsiella. spp.* (Mantel correlation, $r = 0.5874$, $P = 0.001$; Fig. 2D). Altogether, these data indicated that the classification of the esophageal microbial communities differs in ESCC and PN esophagus. Also, our results verify that *Fusobacteria* exists in the ESCC microbiota as a high abundant.

Characterized Microbial Taxa Associated With Esophageal Carcinoma Patients

We used LEfSe analysis to identify the relevant taxa responsible for the significances between clinical diagnoses. 31 taxa, including 10 genera, which was differentially abundant between T and PN groups, was identified. Based on Genus taxa in T groups, the enrichment in *Aggregatibacter*, *Veillonella*, *Parvimonas*, *Catonella*, *Streptococcus*, *Selenomonas*, *Porphyromonas*, *Non-Fusobacterium*. *Fus*, *Lautropia*, *Peptococcus*, *Fusobacterium.spp*, *Peptostreptococcus*, *Campylobacter*, *Dialister*, *Prevotella*, *Treponema*, and *Granulicatella* were observed. In addition, *Treponema amylovorum*, *Streptococcus infantis*, *Prevotella nigrescens*, *Porphyromonas endodontalis*, *Veillonella dispar*, *Aggregatibacter segnis*, *Prevotella melaninogenica*, *Prevotella intermedia*, *Prevotella tannerae*, *Prevotella nanceiensis* and *Streptococcus anginosus* were also significantly more abundant in ESCC by Species taxa (Fig. 3A-C).

The age-matched comparisons of the bacteria taxa in patients with ESCC and physiological normal esophagus was performed by LEfSe analysis (Supplementary figure S3G). To determine the relationships between disease-associated taxa and the abundance of *Fusobacteria*, we subtracted the *Fusobacteria* reads and re-analyzed the library from the dataset by LEfSe analysis. In support of the above, *Streptococcus*, and *Prevotella* in ESCC were enriched (supplementary figure S5). To confirm ESCC-enriched and depleted taxa, we used 16 s rRNA-seq data from a discovery cohort of ESCC. In this dataset, we found significant decreases in the abundance of *Geobacillus*, *Anoxybacillus*, *Thermus*, *Lactococcus*, *Pseudomonas*, *Klebsiella*, *Blastomonas*, and *Acinetobacter* in ESCC compared to physiological normal esophagus (Fig. 3D). To illustrate that our results were not biased by microbiota profiling pipeline, we used a second validation cohort to confirm. In agreement with the results obtained in discovery cohort, the enrichments of *Selenomonas*, *Peptostreptococcus*, *Fusobacterium spp.*, and *Acinetobacter* were confirmed in 19 genera as identified by the LEfSe analysis (Fig. 3E).

EscC Demonstrates Microbial Dysbiosis

We combined the 19 relevant taxa which characterized in patients with ESCC and PN esophagus and estimated the microbial dysbiosis index (MDI). The esophageal microbiota of patients with ESCC had a higher MDI than that patients with PN esophagus both in discovery cohort and validation cohort (Fig. 4A). Moreover, similar results were also observed in age-matched subset of discovery cohort (Supplementary figure S3F). In Fig. 4B and C, we found that MDI had a significant inverse correlation with the alpha diversity ($r = -0.971$, $P < 0.0001$) and a positive correlation with the beta diversity ($r = 0.3956$; $P < 0.001$), indicating that esophageal microbiota has a high degree of dysbiosis, consistent with reduced bacterial diversity. We further assessed if MDI could be use employed to distinguish between ESCC and physiological normal esophagus. By ROC analysis, the MDI had a good performance in identifying ESCC in discovery cohort (AUC = 95.96%, Fig. 4D) and validation cohort (AUC = 93.75%, Fig. 4E). Additionally, The MDI displayed enhanced sensitivity and specificity to monitor ESCC by using single taxa (supplementary figure S6). In validation cohorts (as shown in Fig. 3E), we confirmed the differential abundance of *Fusobacterium* sp., *Peptostreptococcus* sp., *Selenomonas* sp. and *Acinetobacter* sp. We next re-calculated the MDI with these 4 genera. The data showed that microbiota was imbalanced in the discovery cohort, validation cohort and AUCs in the ROC analysis (supplementary figure S7).

Change of nitrate/nitrite reductase functions in the microbiota of ESCC

The microbial connection in ESCC and PN esophagus could be discriminated according to their function (supplementary figure S8A). The predicted KEGG pathways significantly enriched in ESCC included aminoacyl-tRNA biosynthesis, translation proteins, ribosome biogenesis, ribosome, purine metabolism, DNA repair and recombination proteins, DNA replication proteins and Chromosome (supplementary figure S8B and supplementary table S5). Accumulating evidence demonstrated that the microbiota might produce secondary metabolites, such as reactive nitrate and nitrite, which are carcinogens associated with cancer development. We next compared ESCC and PN esophagus regarding the microbial functional signatures involved in nitrate and nitrite reductase (supplementary table S6). The results indicated that the functional composition of ESCC microbiota had decreased nitrate reductase functions and nitrite reductase functions compared to the PN esophagus (Fig. 5A and B). Collectively, these data indicated that the change of nitrate and nitrite reductase functions of ESCC microbiota is present in ESCC.

Discussion

In this study, we profiled the microbial alterations in the tumor and PN tissues of ESCC patients using analyses based on 16S rRNA gene sequencing. We have shown that the microbiota composition in tumor tissues of ESCC patients is significantly different from that of patients with PN tissues. The microbial dysbiosis of ESCC tumor tissues was characterized by decreased microbial diversity, and increased *Bacteroidetes*, *Fusobacteria* and *Spirochactes*. In addition, we also found that the functional features of ESCC microbiota demonstrate reduced nitrate reductase and nitrite reductase functions. Together, our results explored the microbiota spectrum of ESCC patients and revealed the microbial signature associated with ESCC for diagnosis of patients.

The microbiota is less characterized in ESCC than in esophageal adenocarcinoma[20, 32]. Only a few studies have the composition of microbiota on ESCC. Here we focused on the pathological microbiota characterization in ESCC. The composition of the microbiota of the ESCC has been identified in our study. Microbiota studies of esophageal tissues have shown that *Treponema amylovorum*, *Streptococcus infantis*, *Prevotella nigrescens*, *Porphyromonas endodontalis*, *Veillonella dispar*, *Aggregatibacter segnis*, *Prevotella melaninogenica*, *Prevotella intermedia*, *Prevotella tannerae*, *Prevotella nanceiensis* and *Streptococcus anginosus* are enriched in ESCC tissues. Our detection of these 10 bacterial strains are mainly involved in oral health, suggesting that oral hygiene may have roles in tumor development of ESCC. Indeed, lifestyle factors, including diet, obesity, alcohol and tobacco use, and oral hygiene, are involved in high rates of ESCC. In line with this hypothesis, it has been shown that numbers of lost teeth increase the risk of ESCC development[33] and that high burden of *F. nucleatum*, which inhabits the oral cavity and causes periodontal disease, in ESCC correlates with poor RFS [34]. Also, *Porphyromonas gingivalis*, a Gram-negative bacterial species involved in periodontal diseases, is a biomarker of ESCC[35]. In our studies *Fusobacterium nucleatum*, also called by a recently coined name *oncobacterium* because of its association with cancer [36], is again abundant in ESCC tissues of our cohorts. Here our data further characterize top 10 bacteria strains that may influence or directly participate in carcinogenesis and progression of ESCC. *Treponema amylovorum* is a subgingival plaque bacteria species involved in chronic periodontitis[37]. *Streptococcus infantis* is a part of the salivary microbiome [38] and a prevalent bacteria in breast cancer[39]. *Aggregatibacter segnis* is an oral bacterial species in oral cancer[40]. *Porphyromonas endodontalis*, is a part of the salivary microbiome, and its lipopolysaccharide (LPS), which can trigger NFκB signaling [41], is enriched in infected root canals and apical periodontitis [42]. *Veillonella dispar*, a bacterium in oral mucosa, is involved in autoimmune hepatitis[43, 44]. *Streptococcus anginosus* is enriched in gastric cancer[45] and dental implant-related osteomyelitis[46]. *Prevotella intermedia* is a biomarker for periodontal disease[47]. *Prevotella melaninogenica* is a causative agent of periodontitis [48]. *Prevotella nigrescens*, which elicits TLR2 signaling and p65-Mediated Inflammation[49], is an oral bacterial species causing for respiratory tract infections. Further, *Prevotella intermedia*, *Prevotella melaninogenica*, and *Prevotella nanceiensis* are 3 periodontopathic bacteria species causing oral malodor and oral health issues [50]. They have cysteine and serine proteinases activity that may regulate tumorigenesis [50]. Lastly, *Prevotella tannerae*, a periodontopathogenic bacteria, is associated with an increased oral squamous cell carcinoma risk [51]. The resident microbial ecosystem in the esophagus is affected by both oral and gastric bacteria, but our data show that microbiota of ESCC seems to be dominated by oral bacteria. As mentioned above, top enrichments in microbial composition of ESCC are mainly from oral bacteria strains, we conclude that oral microbial dysbiosis leads to the occurrence and development of ESCC. These data suggest that poor oral health is linked to increased risk of developing ESCC. We speculate that these specific bacterial strains in oral samples might be utilized as screening tools to assess the risk for ESCC to identify high risk individuals for more invasive screening procedures (e.g., endoscopy). It is possible that the altered abundances of these bacterial strains have a causative role in ESCC development. Their detailed mechanisms in promoting ESCC require further investigation.

We have depicted the diversity of the gut microbiota in ESCC, but the role of most bacterial species in ESCC remains largely unknown. The complexity of the ESCC microbiota, with a plethora of uncharacterized host-microbe, microbe-microbe, and environmental interactions, contributes to the challenge of advancing our knowledge of the ESCC microbiota-cancer interaction. We hypothesized that the microbes or microbiota contribute to the oncogenic evolution of ESCC. To address the microbiota-cancer interaction, we have investigated KEGG pathways that were enriched in ESCC-associated bacteria. One of the top-ranked KEGG pathway in ESCC microbiota is nitrate reductase function. The nitrate reductase might transform nitrate to nitrite, a precursor of nitrosamines, which are carcinogens associated with ESCC. Nitrate is critical inorganic nitrogen sources for microbes, and many bacteria express assimilatory nitrate reductase (NAS) to catalyze the rate-limiting reduction of nitrate to nitrite. For examples, it is interesting to note that fast-growing environmental mycobacteria carry *nasN*, while slow-growing pathogenic mycobacteria are lacking [52]. Also, it has been shown that nitrite-oxidizing phylum Nitrospirae is reduced in gastric cancer[53], causing decreased nitrate/nitrite reductase functions. These data suggest that nitrate reduction plays role in pathogenic/neoplastic progression. And our observation that reduction of nitrate reductase functions in the microbiota of ESCC may impose pathogenic effects during ESCC progression and development. Given that ESCC-associated taxa can impact the nitrate regulation, it is possible that targeting the microbiota involved in nitrate regulation may be effective and beneficial to ESCC patients.

A recent comprehensive investigation of microbiomes across seven cancer types (not yet including ESCC) indicates that intracellular bacteria are widespread in tumors [39]. Particularly, 19 prevalent bacteria are characterized[39], including genera of streptococcus and fusobacterium, which are found in our ESCC microbiota. It is not clear whether the enriched bacterial species or genera identified in our study can reside in ESCC to facilitate the microenvironment to boost cancer growth. These species may impose immune inflammatory and metabolic burden, and further studies are warranted. ESCC is one of the most aggressive cancers and is therapeutic resistant including radioresistance [54]. The dysbiosis of esophageal microbiota could be the culprit of these impacts. For example, higher *F. nucleatum* burden leads to poor response to neoadjuvant chemotherapy [34], suggesting the possibility that a strategic intervention against this bacterium may significantly improve therapeutic response in patients with ESCC. Our detection of these 10 bacterial strains in ESCC may affect on the efficacy of radiotherapy, chemotherapy, or immune checkpoint inhibitor therapy. Indeed, microbiota composition can influence the treatment efficacy of immune checkpoint inhibitor in melanoma[55, 56]. As immune checkpoint inhibitors (e.g., nivolumab, or pembrolizumab) are being used to treat ESCC[57], unraveling ESCC microbiota-drug interactions and efficacy warrants further investigation.

Conclusion

In summary, our studies suggest that ESCC-specific microbial taxa may serve as sensitive and specific clinical diagnostic markers. It is possible that targeting these bacterial strains may be effective and beneficial to ESCC patients. Correct microbial assessment will aid in the detection and treatment of ESCC in the future.

Abbreviations

ESCC
esophageal squamous cell carcinoma; PN:physiological normal; CRC:colorectal cancer; NGS:next-generation sequencing; OTUs:operational taxonomic units; PCoA:principle coordinate analysis; LDA:linear discriminant analysis; LEfSe:linear discriminant analysis effect size; MDI:microbial dysbiosis index; LPS:lipopolysaccharide; KEGG:Kyoto Encyclopedia of Genes and Genomes; NAS:nitrate reductase; NSTI:Nearest Sequenced Taxon Index; ROC:receiver operating characteristic; AUC:area under the curve;

Declarations

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Authors contributions

Study design: C.C, M.H.L; Sample collection and processing: W.X.Y, X.B.X, Z.F.Z, Z.G.L, B.Z; Clinical data collection and interpretation: W.X.Y, C.H.C, M.H.J, Z.F.Z, Z.G.L, B.Z. Bioinformatic analysis and statistics: W.X.Y, C.H.C, M.H.J, Z.F.Z, L.G; Manuscript preparation: W.X.Y, C.H.C, L.G, S.J.Y, C.C, M.H.L; Approval of final draft submission: W.X.Y, C.H.C, M.H.J, X.B.X, L.G, Z.F.Z, Z.G.L, B.Z, S.J.Y, M.H.L, C.C.

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Availability of data and materials

The sequencing dataset analyzed during the current study will be available, upon publication (<https://pan.genomics.cn/ucdisk/s/Yn2eQ3>). All other data is available from the corresponding author.

Ethics approval and consent to participate

This study was conducted according to a protocol approved by the respective Institutional Ethics Committees of the First Affiliated Hospital of Sun Yat-sen University and according to the Declaration of Helsinki. Written informed consent was obtained from all participants.

Consent for publication

Written informed consent was obtained from all participants.

Competing interests

The authors declare no competing interests.

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Figures

Figure 1

The profile of esophageal microbiota differs in the PN and T groups. (A) Number of OTUs in PN and T groups, measuring the total bacterial species diversity in two groups. (B) Good's coverage Index of diversity, measuring the data coverage rate of total bacterial species in two groups. (C) Principal coordinate analysis (PCoA) plots of unweighted UniFrac distances between PN and T groups, including ANOSIM analysis of similarity. (D) Principal coordinate analysis (PCoA) plots of weighted UniFrac distances between PN and T groups, including ANOSIM analysis of similarity. PN represents healthy group; T represents tumor groups.

Figure 2

The abundance of *Fusobacterium spp.* affects the microbiota composition of PN and T groups. (A) Relative abundance of phyla in all samples and in each group. (B) Spearman's rank correlation between relative abundance of *Fusobacterium spp.* and *Klebsiella spp.* in all samples. (C) Principal coordinate analysis (PCoA) plots of the weighted UniFrac distance displayed by increasing the relative abundance of *Fusobacterium spp.* between PN and T groups, including Mantel correlation analysis. (D) Principal coordinate analysis (PCoA) plots of weighted UniFrac distances displayed by increasing the relative abundance of *Klebsiella spp.* between PN and T groups, including Mantel correlation analysis.

Figure 3

Characterized microbial taxa associated with esophageal carcinoma patients. (A) GraPhlAn (Graphical Phylogenetic Analysis) result of the esophageal microbial biomarker between PN and T groups. (B) Genus microbial biomarker between PN and T groups by linear discriminant analysis (LDA) effect size (LEfSe). (C) Species microbial biomarker between PN and T groups by linear discriminant analysis (LDA) effect size (LEfSe). Green represents genus biomarker enriched in PN group and red represents genus biomarker enriched in T group. (D) Relative abundance of the 19 genera differentially enriched between PN and T groups in the Discovery cohort. $P < 0.05$ significance obtained by Metastats software analysis. (E) Relative abundance of the 19 genera differentially enriched between PN and T groups in the Validation cohort. $P < 0.05$ significance obtained by Metastats software analysis.

Figure 4

Esophageal carcinoma demonstrates microbial dysbiosis. (A) Box plot displaying the MDI in the discovery and validation cohorts. P-value was obtained by Wilcoxon test for comparisons from two groups. (B) Negative Pearson's correlation between MDI and Number of OTUs. (C) Principal coordinate analysis (PCoA) plot of weighted UniFrac distance showed by increasing MDI between PN and T groups. Mantel correlation analysis with 999 permutations were used. (D, E) ROC curves analysis to assess the discriminatory potential of MDI in the discovery and validation cohorts. MDI, microbial dysbiosis index; ROC, receiver operating characteristic; AUC, area under the curve.

Figure 5

Change of nitrate/nitrite reductase functions in the microbiota of ESCC. (A) COG functional analysis of the predicted metagenome of the microbiota between PN and T groups from the discovery cohort. (B) KO functional analysis of the predicted metagenome of the microbiota between PN and T groups from the discovery cohort. The normalized relative frequency of nitrate reductase and nitrite reductase in two groups. $P < 0.05$ significance obtained by STAMP software analysis. COG, Clusters of Orthologous Groups;

KO, Kyoto Encyclopedia of Genes and Genome (KEGG) orthology; NADH, nicotinamide adenine dinucleotide; NO, nitric oxide; MAO, trimethylamine N-oxide.

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