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Comparison of tumor-associated and nontumor-associated esophageal mucosa microbiota in patients with esophageal squamous cell carcinoma

Baihua Zhang, MD[a], Qin Xiao, MD[b], Huahai Chen, PhD[c], Tao Zhou, PhD[c], Yeshi Yin, PhD[c,*]

Abstract

Esophageal microbiota plays important roles in esophageal squamous cell carcinoma (ESCC). The aims of this study were to clarify the changes in the bacterial community during ESCC development and identify latent pathogenic bacteria which may contribute to esophageal carcinogenesis and progression. Fresh tumor and nontumor esophageal mucosal samples were collected from 31 men with ESCC. High-throughput 16s rRNA sequencing was performed, and the operational taxonomic unit data and bacterial classification annotation were obtained and analyzed. The Ace, Chao, Shannon, Simpson indexes, and operational taxonomic unit numbers were higher in nontumor tissues than in tumor tissues, although without statistical significance. There were 4 phyla and 28 genera found to show significant differences between tumor and nontumor samples. The general probiotic *Lactobacillus* was 1.98-fold higher in nontumor tissues, while the general pathogenic genera *Fusobacterium* was 4.35-fold higher in tumor tissues. For tumor tissue samples, the genera *Treponema* and *Brevibacillus* were significantly higher in N1 and N2 stages, respectively, and *Acinetobacter* was significantly higher in T3 stage. For nontumor tissues, the genus *Fusicatenibacter* was significantly higher in T2 stage, and *Corynebacterium*, *Aggregatibacter*, *Saccharimonadaceae*-TM7x, and *Cupriavidus* were significantly higher in T4 stage. Additionally, bacteria related to nitrotoluene degradation were enriched in nontumor tissues, while bacteria related to base excision repair were enriched in tumor tissues. The relative abundance of several phyla and genera are different between tumor and nontumor tissue samples. The altered bacterial microbiota is correlated with different tumor stages and some microbes may take part in the carcinogenesis and development of ESCC.

Abbreviations: ESCC = esophageal squamous cell carcinoma, LEfSe = linear discriminant analysis Effect size, OTU = the operational taxonomic unit, PICRUSt = phylogenetic investigation of communities by reconstruction of unobserved States.

Keywords: 16s rRNA high-throughput sequencing, carcinogenesis, Esophageal squamous cell carcinoma, microbiota, tumor tissue.

1. Introduction

Esophageal carcinoma is one of the most common malignant tumors and ranked seventh in terms of incidence and sixth in overall mortality worldwide.[1] Nearly half of the newly diagnosed esophageal cancer cases were in China, and approximately 90% of these were esophageal squamous cell carcinoma (ESCC).[2,3] However, the etiology of ESCC is poorly understood. Previous investigations have indicated that the major and potential risk factors for ESCC include tobacco smoking, alcohol consumption, hot drinks, male sex, lower body mass index, and intrinsic esophageal diseases.[2–4] In recent years, several lines of evidence have indicated that the human microbiome is a potential player in carcinogenesis and tumor progression.[5] It has been reported that several pathogenic microorganisms can promote the occurrence and development of tumors, including gastric cancer, colon cancer, liver cancer, and pancreatic cancer.[6] However, there is little research on the microbial environment of ESCC.

It has been reported that approximately 15.4% of all cancer cases are attributable to infections, such as gastric cancers caused by *Helicobacter pylori* and cervical cancer caused by

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The datasets generated during and/or analyzed during the current study are publicly available.

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human papillomavirus.[7] Recently, the role of gut microbiota in cancer microenvironmental factors has become a hotspot. Gut microbiota could affect both the local immunity of the digestive tract mucosa and systemic immune response of the whole body, which might lead to consistent chronic inflammation and contribute to cancer.[8] In the case of the esophagus, Yu et al[9] reported that lower microbial richness was associated with the presence of esophageal squamous dysplasia, which was a precursor for the majority of ESCC cases. Gao et al[10] found that *Porphyromonas gingivalis* could only be detected in esophageal tissues of ESCC, and that the load of *P gingivalis* was positively associated with the progression and poor prognosis of ESCC. Other studies reported that ESCC tumor tissues contained more Fusobacterium and less Streptococcus, and that high levels of Fusobacterium nucleatum in the tumor were associated with greater resistance to neoadjuvant chemotherapy treatment and poor prognosis of ESCC.[11,12] Moreover, antibiotic treatment could negatively modulate the chemotherapeutic efficacy in patients with esophageal cancer due to microbiota dysbiosis.[13] Taken together, increasing evidence suggests that esophageal microbiota may contribute to development and therapeutic efficacy of esophageal cancer. Therefore, there are unprecedented opportunities to identify new microbial biomarkers for the diagnosis and treatment of esophageal cancer.

The aims of the present study were to clarify the changes in the bacterial community during ESCC development and identify latent pathogenic bacteria which may contribute to esophageal carcinogenesis and progression.

2. Materials and Methods

2.1. Patient eligibility and evaluation

Considering the different risk factors to which both sexes and the various age groups might be exposed, only male patients aged > 50 years were recruited into the study to avoid any sex- and age-specific biases. In total, thirty-one male patients with ESCC were included. All patients received curative esophagectomy at Hunan Cancer Hospital (Changsha, China) between October 2018 and July 2019, and no other antitumor therapy was performed prior to surgery. The pathological TNM stage was classified according to the eighth edition of the American Joint Committee on Cancer Staging System for esophageal carcinoma. All procedures were approved by the Ethics Committee of Hunan Cancer Hospital (2021043), informed consent was obtained from each participant. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Esophageal mucosa samples were aseptically isolated within 15 minutes after the specimens were removed from the body. Both primary tumor and paired nontumor tissues (esophageal mucosa at least 5 cm above the primary tumor as the control group) were collected, and then quickly frozen in liquid nitrogen for further analysis.

2.2. Bacterial genome DNA extraction

Tissue samples were cut into small pieces, and bacterial genomic DNA was then extracted using QIAamp Fast DNA Stool Mini Kits (Qiagen, Germany) according to the manufacturer's instructions. As an option in the kit instructions, we used the FastPrep-24 combined bead-beating method to promote bacterial lysis and improve the DNA acquisition rate. The DNA concentration was measured using a NanoDrop2000 (Thermo, Thermo scientific TM, USA).

2.3. High-throughput 16S rRNA sequencing

Bacterial genomic DNA samples were sent to Hangzhou Lizhen Biomedical Technology Co., Ltd, Hangzhou, China. for high-throughput sequencing using the Illumina MiSeq platform. The primers used for amplification of the 16S rRNA gene V3 to V4 region were as follows: 338F (5′-ACT CCT ACG GGA GGC AGC A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′). The basic information relating to the high-throughput sequencing is listed in Table S2 (Supplemental Digital Content 1, http://links.lww.com/MD/H239). The raw sequence data obtained in this study were submitted to the NCBI database under accession number PRJNA751695 (https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA751695).

2.4. Bacterial community analysis

The obtained sequences were first analyzed using QIIME[14,15] and Mothur[16,17] software and classified by operational taxonomic unit (OTU) using 97% similarity as the classification threshold. The representative sequences of each OTU were annotated using the Ribosomal Database Project classifier and the Silva database. The OTU data and bacterial classification annotation obtained were then analyzed using the bacterial diversity analysis software package (http://amplicon.vgenomics.cn:9000/) developed by Shanghai Yingfei Biotechnology Co., Ltd, Shanghai China. The analysis module includes α diversity analysis, β diversity analysis, bacterial community analysis, linear discriminant analysis Effect size (LEfSe) analysis, and Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) bacterial gene function prediction analysis. All these analyses were conducted using the default parameters of the software.

2.5. Statistical analysis

Bacteria that were significantly different between or among groups were identified by LEfSe software. The paired sample *t* test included in the SPSS statistical software package (SPSS V.20.0; IBM Corp., NY, USA) was used for significant difference kyoto encyclopedia of genes and genomes analysis. All *P* values were 2 tailed, and a P value < .05 was considered statistically significant.

3. Results

3.1. Patient characteristics

All the participants were from Hunan province, an area in the center of China, where residents had similar dietary habits and lifestyles. The baseline information of these participants is listed in Table S1 (Supplemental Digital Content 2, http://links.lww.com/MD/H240). The mean age of the group was 64.94 ± 7.24 (range, 50–78) years. No family history of esophageal carcinoma was observed in this group. Progressive dysphagia was the principal manifestation (100%) at the initial diagnosis. The primary lesions were most located in the middle and lower thoracic esophagus (29 cases, 93.6%). Twenty-five patients (80.6%) had a history of smoking, and 26 patients (83.9%) had a history of alcohol exposure. The median tumor length was 4.6 cm (range, 2.0–7.5 cm).

No patient in the group received neoadjuvant therapy before surgery, and no antibiotics were used within 1 month prior to surgery. All the patients underwent partial esophagectomy and regional lymph node dissection through 2 or 3-incisional thoracotomy, and radial resections with stomach as the esophageal substitute were achieved in all cases. After surgery, 16 cases (51.6%) were identified with well-differentiated ESCC, 11 cases (35.5%) with moderately differentiated ESCC, and 4 cases (12.9%) with poorly differentiated ESCC. Six cases (19.3%) had lesions in pathological stage T2, 14 cases had lesions in pathological stage T3 (45.2%), and 11 cases had lesions in pathological stage

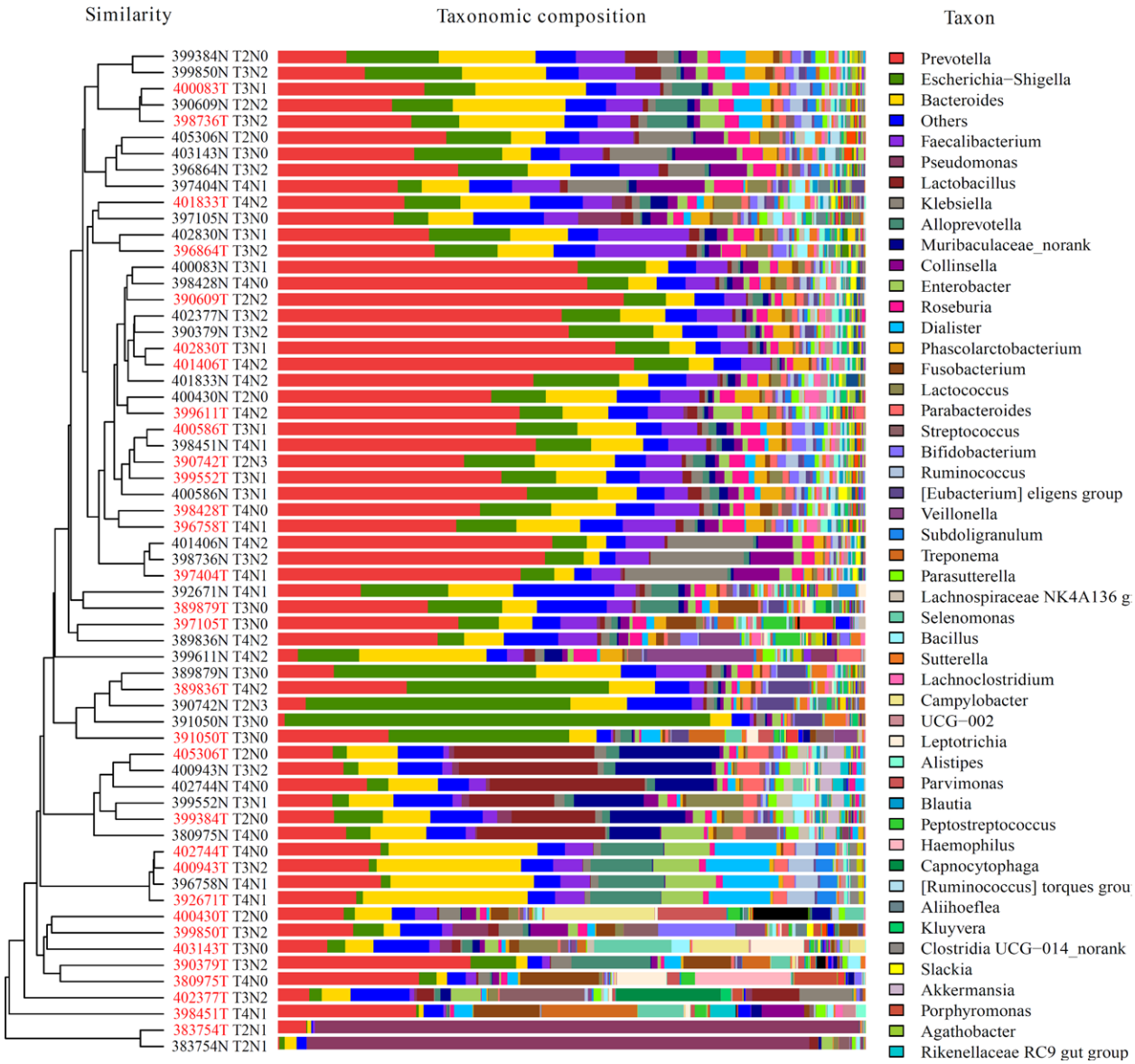

Figure 1. Bacterial community and cluster analysis of esophageal mucosal samples. The software of Visual Genomics 1.4.1 (Shanghai InfinityBio Technology Co. Ltd) was used to generate this picture. The numbers at the right side of the cluster tree represent patient numbers. N or T after the patient numbers represent nontumor or tumor samples, respectively. T(2-4) N(0-3) represents different tumor stages.

T4 (35.5%). For N stage classification, 10 cases (32.3%) were classified as stage N0, 9 cases were classified as stage N1 (29.0%), and 12 cases were classified as stage N2-N3 (38.7%). For TNM stage classification, there were 7 cases (22.6%) in stage IIA-B, 19 cases (61.3%) in stage IIIA-B, and 5 cases (16.1%) in stage IVA.

3.2. Barcode 16s rRNA pyrosequencing and diversity analysis

For all 31 patients, 62 tissue samples were collected for bacterial genome DNA extraction and 16S rRNA V3-V4 region high-throughput sequencing. As indicated in Table S2 (Supplemental Digital Content 1, http://links.lww.com/MD/H239), 46, 963 clean reads were obtained for these samples at an average level. Although slightly higher levels of Ace, Chao, Shannon, and Simpson indices were detected in nontumor tissues than in tumor tissues, the difference was statistically insignificant (data not shown).

3.3. Microbial composition of esophageal mucosal tissues

At the bacterial phylum level, 39 phyla were detected in all samples. Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, and Fusobacteria were the top 5 phyla detected in these samples (Figure S1, Supplemental Digital Content 3, http://links.lww.com/MD/H241), which accounted for 42.15%, 28.57%, 21.75%, 3.68%, and 1.69%, respectively. Phyla of Actinobacteria, Bacteroidetes, Desulfobacterota, Firmicutes, Fusobacteria, and Proteobacteria were detected in all tumor and nontumor tissues.

At the bacterial genus level, a total of 899 genera were identified. As illustrated in Figure 1, *Prevotella*, *Escherichia-Shigella*, *Bacteroides*, *Faecalibacterium*, *Pseudomonas*, *Lactobacillus*, *Klebsiella*, *Alloprevotella*, *Collinsella*, and *Enterobacter* were the top 10 genera identified in these samples, which accounted for 25.70%, 10.56%, 9.02%, 4.95%, 3.37%, 2.89%, 2.45%, 2.07%, 1.90%, and 1.87%, respectively. These genera, which were detected in all samples, were considered core genera. As summarized in Table 1, 37 and 34 genera were identified as core

Table 1

Relative abundance of core genera detected in tumor and nontumor tissue samples.

Genus	Nontumor	Tumor	Nontumor/tumor
Aliihoeflea	0.35%	N	UN
Alistipes	0.40%	0.39%	1.03
Alloprevotella	1.29%	2.86%	0.45
Bacillus	0.55%	N	UN
Bacteroides	9.01%	9.02%	1.00
Barnesiella	N	0.12%	UN
Bifidobacterium	0.64%	1.05%	0.61
Blautia	0.42%	0.32%	1.33
Colidextribacter	0.10%	N	UN
Collinsella	2.48%	1.31%	1.90
Enterobacter	1.67%	2.06%	0.81
Escherichia-Shigella	12.97%	8.15%	1.59
[Eubacterium] coprostanoligenes group_norank	0.18%	N	UN
[Eubacterium] eligens group	0.90%	N	UN
Faecalibacterium	5.46%	4.43%	1.23
Fusobacterium	0.45%	1.99%	0.23
Klebsiella	3.04%	1.87%	1.62
Kluyvera	0.31%	0.29%	1.04
Lachnoclostridium	0.58%	0.42%	1.38
Lachnospiraceae NK4A136 group	0.65%	0.47%	1.38
Lachnospiraceae_uncultured	0.30%	0.22%	1.39
Lactobacillus	3.84%	1.94%	1.98
Lactococcus	1.17%	1.06%	1.10
Monoglobus	0.17%	N	UN
Muribaculaceae_norank	2.26%	1.64%	1.38
Oscillospiraceae_UCG-002	0.49%	0.48%	1.02
Oscillospiraceae_UCG-003	0.25%	0.29%	0.88
Oscillospiraceae_UCG-005	0.20%	0.15%	1.37
Parabacteroides	1.23%	0.95%	1.30
Parasutterella	0.71%	0.48%	1.49
Phascolarctobacterium	1.79%	1.28%	1.39
Prevotella	25.11%	26.28%	0.96
Prevotellaceae_uncultured	N	0.05%	UN
Roseburia	2.08%	1.53%	1.36
Ruminococcus	0.69%	0.94%	0.73
[Ruminococcus] torques group	0.35%	0.27%	1.32
Slackia	N	0.23%	UN
Streptococcus	0.70%	1.03%	0.69
Subdoligranulum	0.61%	0.74%	0.82
Sutterella	0.63%	0.39%	1.63

N = not core genus, UN = undone.

microbes for nontumor and tumor tissues, respectively. The percentage of core microbes accounted for 84.03% and 74.70% in nontumor and tumor tissues, respectively. Interestingly, the general probiotic genera *Lactobacillus* was 1.98-fold higher in nontumor tissues than in tumor tissues. In contrast, the general pathogenic genera *Fusobacterium* was 4.35-fold higher in tumor tissues than in nontumor tissues (Table 1 and Figure S2, Supplemental Digital Content 4, http://links.lww.com/MD/H242).

At the OTU level, the individual differences were large, ranging from 121 to 1077 OTUs, with an average of 442 OTUs (Table S2, Supplemental Digital Content 1, http://links.lww.com/MD/H239). Although a slightly higher OTU number was detected in nontumor tissues than in tumor tissues, no significant difference was detected.

3.4. Difference in esophageal mucosa microbiota between tumor and nontumor tissues

Beta diversity analysis (data not shown) and cluster tree analysis (Fig. 1 and Figure S1, Supplemental Digital Content 3, http://links.lww.com/MD/H241) indicated that the samples did not cluster together according to their original sampling site, and that tumor and nontumor tissues did not cluster together at the whole microbiota level. In addition, samples did not cluster together according to patients' T or N staging. However, some bacteria were found to be significantly different between tumor and nontumor tissues (Fig. 2). LEfSe analysis showed that 2 phyla, 1 class, 2 orders, 3 families, and 13 genera were significantly higher in tumor tissues; and that 2 phyla, 2 classes, 5 orders, 12 families, and 15 genera were significantly higher in nontumor tissues. At the genus level, the genera of *Alloprevotella*,

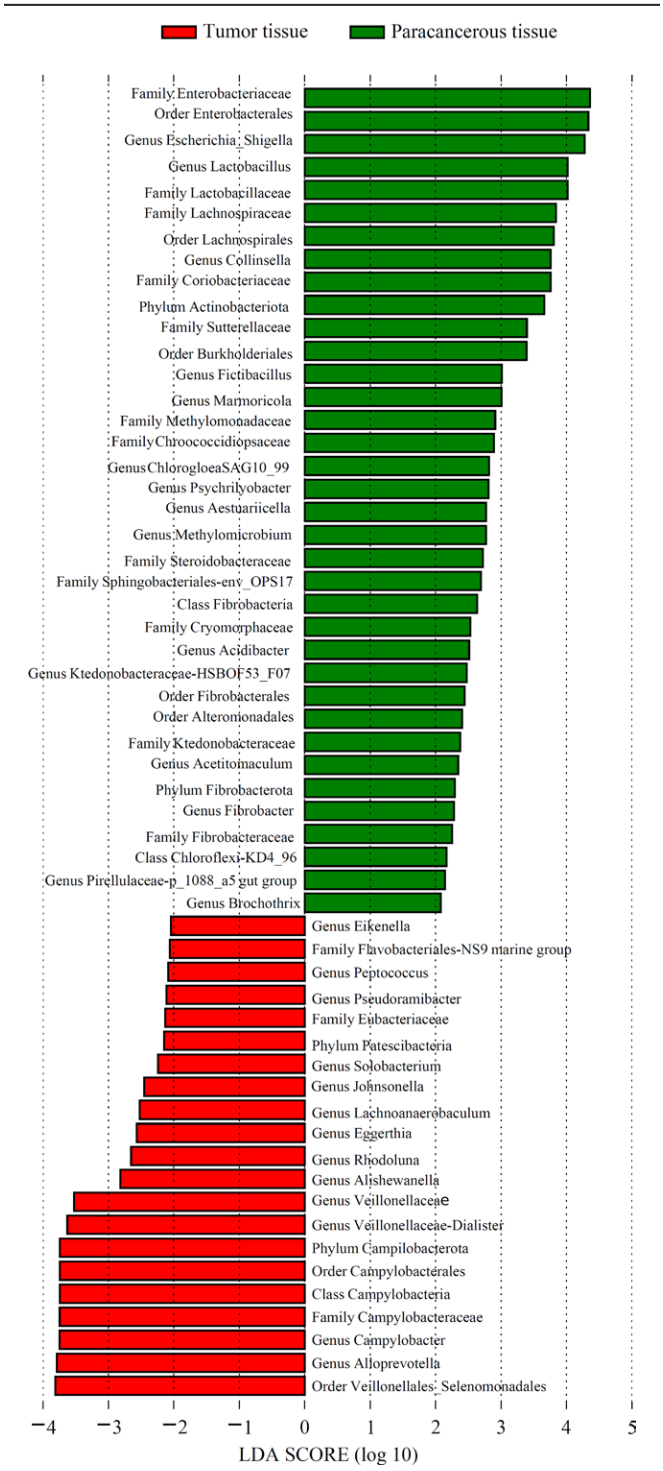

Figure 2. Significant differences in bacterial taxa between tumor and nontumor samples using LEfSe analysis. The LEfSe included in the software of Visual Genomics 1.4.1 (Shanghai InfinityBio Technology Co. Ltd) was used to generate this picture.

Camphlobacter, *Veillonellaceae-Dialister*, *Veillonellaceae*, *Alishewanella*, *Rhodoluna*, *Eggerthia*, *Lachnoanaerobaculum*, *Johnsonella*, *Solobacterium*, *Pseudoramibacter*, *Peptococcus*, and *Eikenella* were significantly higher in tumor tissue samples; in contrast, the genera of *Escherichia_Shigella*, *Lactobacillus*, *Collinsella*, *Fictibacillus*, *Marmoricola*, *ChlorogloeaSAG10_99*, *Psychrilyobacter*, *Aestuariicella*, *Methylomicrobium*, *Acidibacter*, *Ktedonobacteraceae-HSBOF53_F07*, *Acetitomaculum*, *Fibrobacter*, *Pirellulaceae-p_1088_a5* gut group, and *Brochothrix* were significantly higher in the nontumor tissue samples.

3.5. Differences in predicted metabolic pathways between nontumor and tumor tissues

The PICRUSt software was selected in this study to predict the metagenomes from the bacterial 16S rRNA gene high-throughput sequencing data, and the predicted kyoto encyclopedia of genes and genomes pathways were used for comparative analysis. As illustrated in Figure S3 (Supplemental Digital Content 5, http://links.lww.com/MD/H243), pathways related to transport, general function prediction only, ABC transporters, DNA repair and recombination proteins, purine metabolism, ribosome, peptidases, transcription factors, pyrimidine metabolism, and 2-component system were the top 10 metabolic pathways predicted for these bacteria. The composition of these top 10 pathways in tumor and nontumor tissues was almost the same level (Figure S3, Supplemental Digital Content 5, http://links.lww.com/MD/H243). However, more detailed analysis indicated that the pathway of base excision repair was significantly higher in tumor tissues (Fig. 3A), while the pathway of nitrotoluene degradation was significantly higher in nontumor tissues (Fig. 3B).

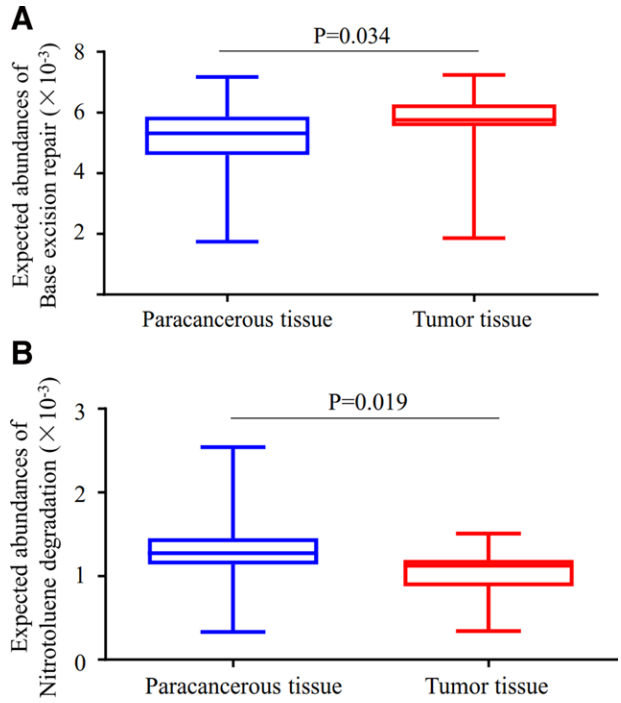

Figure 3. Predicted significantly different pathways of the esophageal microbiota between tumor and nontumor tissues using PICRUSt software. (A) The pathway of base excision repair was significantly higher in tumor tissues. (B) The pathway of nitrotoluene degradation was significantly higher in nontumor tissues. GraphPad Prism 8.0.1 (GraphPad Software) was used to generate this graph.

3.6. Changes in mucosal microbiota in different stages of cancer

As seen in Table S1 (Supplemental Digital Content 2, http://links.lww.com/MD/H240), 6, 14, and 11 patients were classified into T2, T3, and T4 stages, respectively; 10, 9, and 11 patients were classified into N0, N1, and N2 stages, respectively. To investigate the changes in the bacterial flora in different stages of cancer differentiation, the LEfSe software was selected to perform further analysis of these samples.

Compared to nontumor tissue samples, more significant bacteria were detected at different N stages among tumor tissues (Fig. 4 and Figure S4, Supplemental Digital Content 6, http://links.lww.com/MD/H244). For tumor tissue samples, at the genus level, *Peptostreptococcaceae*, *Leptotrichia*, *Peptostreptococcus*, *Anaerovoracaceae*, *Filifactor*, *Anaerovoracaceae-Eubacterium_brachygroup*, *Lachnoanaerobaculum*, *Dethiosulfatibacteraceae*, *Solobacterium*, *Johnsonella*, *Prevotellaceae* UCG_001, and *Tannerella* were significantly higher in N0 stage; and *Treponema* and *Brevibacillus* were significantly higher in N1 and N2 stages, respectively. For T stage analysis, the genus *Acinetobacter* was significantly higher in T3 stage tumor tissues (Figure S5A, Supplemental Digital Content 7, http://links.lww.com/MD/H245); the genera *Corynebacterium*, *Aggregatibacter*, *Saccharimonadaceae*-TM7x, and *Cupriavidus* were significantly higher in T4 stage nontumor tissues; and the genus *Fusicatenibacter* was significantly higher in T2 stage nontumor tissues (Figure S5B, Supplemental Digital Content 8, http://links.lww.com/MD/H246).

4. Discussion

Increasing evidence supports the claim that the gut microbiota mediates the carcinogenesis, development, and therapeutic efficacy of esophageal cancer.[18] The gut microbiota principally refers to the microorganisms that dwell in the oral cavity, esophagus, and intestine.[19] Previous studies have reported that the microbiota in oral and fecal samples in patients with esophageal cancer might exhibit a shift relative to that in healthy individuals, and these changes may be helpful in the early diagnosis of esophageal cancer.[20–24] However, the altered bacterial microbiota observed in oral and fecal samples only had an indirect correlation with esophageal cancer, and the underlying mechanism in carcinogenesis and tumor progression remained undetermined. In the present study, esophageal mucosa samples were analyzed and significant differences in esophageal microbiota between tumor and nontumor tissues were detected, and several latent pathogenic bacteria which may contribute to esophageal tumorigenesis and progression were also identified. There results may lay a solid foundation for revealing the vital role of microbiota in ESCC.

Esophageal carcinogenesis is a complicated course affected by both genetic and environmental factors. Many genome-wide association studies have shown that the number of single-nucleotide polymorphisms at or close to the HLA2 region, including *PLCE1*, *PDE4D*, *RUNX1*, *TMEM173*, *ATP1B2*, *CASP8*, and *ALDH2* genes, are associated with ESCC, but their application in clinical practice is rare.[25–28] With the continuous revelation of the function of microbial flora, the disorder of microbial flora has become a new target for disease diagnosis and treatment. Some studies have shown that lower microbial richness in the upper digestive tract is associated with esophageal squamous dysplasia (a precursor lesion of ESCC).[9] Moreover, Li et al[29] indicated that the microbial diversity was significantly lower in patients with ESCC compared to that in the healthy control group. Yang et al[30] also reported that the ESCC microbiota was characterized by reduced microbial diversity, and decreased abundance of Bacteroidetes, Fusobacteria, and Spirochaetes. Our study also observed a slight reduction in Ace, Chao, Shannon, and Simpson indexes, and OTU numbers in tumor

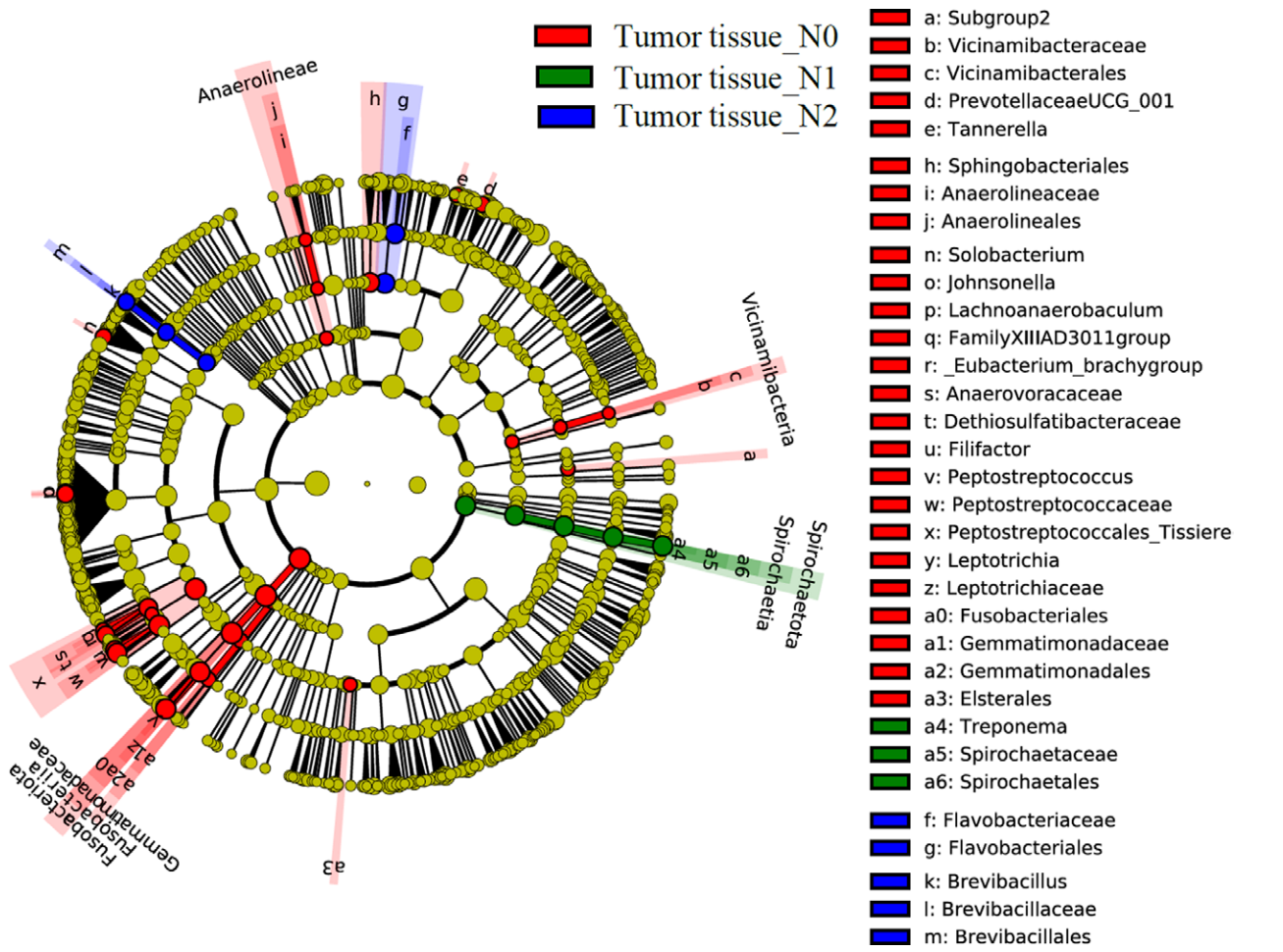

Figure 4. Significant differences in bacterial taxa among different N stages using LEfSe analysis. The LEfSe included in the software of Visual Genomics 1.4.1 (Shanghai InfinityBio Technology Co. Ltd) was used to generate this picture.

tissues compared to nontumor tissues, although the difference was statistically insignificant.

For bacterial community analysis, LDA coupled with LEfSe analysis showed that 4 phyla were found the key taxa contributing to the changes in the microbiota of patients with ESCC. Moreover, the phyla *Actinobacteria* and *Fibrobacteria* are higher in nontumor samples, while *Patescibacteria* and *Campilobacteria* are higher in tumor samples. In addition to these LEfSe analysis results, the phylum Fusobacteria was also found to be significantly higher in tumor tissues than in nontumor samples according to the results of a paired sample *t* test (Figure S2, Supplemental Digital Content 4, http://links.lww.com/MD/H242). These results were consistent with the study by Li et al,[29] who found that the abundance of the phylum Fusobacteria was higher and that of Actinobacteria was lower in the ESCC group than in the healthy group.

At the genus level, previous studies have shown that the esophagus of patients with ESCC was enriched in Streptococcus, Prevotella, Fusobacterium, Veillonella, Clostridiales, Pseudomonas, and Lactobacillus compared to the healthy control group, while Haemophilus, Neisseria, and Porphyromonas showed a decreasing tendency.[11,31,32] However, variations in esophageal microbiota were also found among these studies.[11,29,31,32] Twenty-eight genera were found to be the key taxa in the present study. Although most of these genera were not consistent with previous studies, the general pathogenic genera Fusobacterium was 4.35-fold higher in tumor tissues than in nontumor tissues, while the general probiotic genera Lactobacillus was 1.98-fold higher in nontumor tissues, which is consistent with previous results.[11,12,33,34] Yamamura et al[12] demonstrated that a high level of Fusobacterium in the tumor was associated with a larger tumor size, higher T stage, and higher TNM stage. Li et al[33] reported that Fusobacterium nucleatum was closely related to the pT stage and clinical stage of ESCC. In another study, the compositions of microbes Fusobacteriales, Lactobacillus, Clostridiales, Proteobacteria, and Negativicutes were correlated with the clinical characteristics of esophageal cancer.[34] Our study also observed certain correlations between other microbes and tumor stages. For tumor tissue samples, at the genus level, Peptostreptococcaceae, Leptotrichia, Peptostreptococcus, Anaerovoracaceae, Filifactor, Anaerovoracaceae-Eubacterium_brachygroup, Lachnoanaerobaculum, Dethiosulfatibacteraceae, Solobacterium, Johnsonella, Prevotellaceae UCG_001, and Tannerella were significantly higher in N0 stage tumor tissues; and Treponema and Brevibacillus were significantly higher in N1 and N2 stages. For T stage analysis, the genus Acinetobacter was significantly higher in T3 stage tumor tissues; the genera Corynebacterium, Aggregatibacter, Saccharimonadaceae-TM7x, and Cupriavidus were significantly higher in T4 stage nontumor tissues; and the genus Fusicatenibacter was significantly higher in T2 stage nontumor tissues. However, due to the limited sample size and the absence of prognostic information, no accurate conclusions could be drawn in the present study. However, such correlations suggest that some microbes might play important roles in the carcinogenesis and development of esophageal cancer and are worthy of further investigation.

Considering gene redundancy, it may be more valuable to study the relationship between microorganisms and ESCC at the

functional gene level. We also attempted to use mucosal tissue or bacteria that were washed off from the tissues for metagenomic sequencing. Unfortunately, human genomic DNA pollution is commonplace, and as a result, the available microbial gene information is limited. Therefore, we selected the software PICRUSt to predict and analyze the microbiome function of mucosal tissue in this study. The results of PICRUSt showed that the pathways related to base excision repair were significantly higher in tumor tissues, while those related to nitrotoluene degradation were significantly higher in nontumor tissues. Li et al[29] also reported that pathways related to other cellular functions, including DNA repair and recombination were enriched in the ESCC group. Previous studies[35,36] have suggested that a high level of nitrate in drinking water increases the risk of ESCC, possibly through the formation of N-nitroso compounds. The finding that pathways of nitrotoluene degradation was significantly higher in nontumor tissues may partially explain this observation. Most of the nitrogen-related carcinogens may be degraded in normal tissues. However, this discovery requires a more detailed molecular experiment to verify.

There are several limitations to this study. First, the sample size was limited, and only male patients aged > 50 years old were included in this study. Although the incidence of ESCC in males was significantly higher than that in females, there remain numerous females with ESCC considering the large population in China; thus, there is need to include female patients in future studies. Second, the shot-gun microbiome data were not obtained. Third, more multicenter, large population tests and molecular mechanism experiments are needed to verify these findings and hypotheses.

5. Conclusions

In conclusion, we compared the microbial composition of the esophagus mucosa between tumor and nontumor tissue samples of patients with ESCC. The relative abundance of 4 phyla and 28 genera are different between tumor and nontumor tissue samples. The altered bacterial microbiota is correlated with different tumor stages. Bacteria related to nitrotoluene degradation were enriched in nontumor tissues, while those related to base excision repair were enriched in tumor tissues. Such findings might lay a foundation for further study of the molecular mechanism of bacteria in the occurrence and development of ESCC and offer an opportunity for developing new methods for the prevention and treatment of ESCC.

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