Original Article

Distribution of esophagus flora in esophageal squamous cell carcinoma and its correlation with clinicopathological characteristics

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Background: Esophageal cancer is one of the most common malignant tumors in the digestive system in China. However, the specific pathogenic factors and mechanisms of esophageal cancer are not yet clear. Here, the distribution of esophageal flora in esophageal squamous cell carcinoma (ESCC) and its correlation with clinicopathological characteristics are analyzed.

Methods: Fifty-four patients with ESCC diagnosed in our hospital from June 2018 to January 2020 were selected. The patients' gender, age, course of the disease, the grade of pathological tissue, and degree of differentiation were recorded. The distribution of esophageal mucosa flora in patients with ESCC and significant esophageal flora in the esophageal mucosa of different patients were compared.

Results: At the genus level, Proteus, Firmicutes, Bacteroides, and Fusobacterium are the main dominant bacteria in esophageal cancer tissues. At the subordinate level, Prevotella, Clostridia, Streptococcus, Delftia, Klebsiella, Serratia, and some unclassified florae belong to the dominant species. Furthermore, there were no significant differences in the abundance of bacteria between the esophageal cancer tissues and the normal cancerous tissues ($P>0.05$). Also, there was no difference in the diversity of bacterial flora in ESCC tissues of different parts, different morphology, different staging, and different lymph node metastasis ($P>0.05$). The abundance of Firmicutes, Proteobacteria, and Bacteroides was significantly higher than Clostridia. Furthermore, Actinobacteria and Spirochaetae had the lowest abundance of Spirochaetae. The abundance of Spirochaetae of ulcerative type was significantly higher than medullary type ESCC, while the abundance of Actinobacteria of both medullary and ulcerative types was significantly lower than other types ($P<0.05$). There were no significant differences in esophageal flora abundance in different tumor stages of esophageal cancer mucosal tissues ($P>0.05$). The abundance of Proteobacteria was significantly reduced with the presence of lymph node metastasis, while Bacteroides abundance increased significantly ($P<0.05$).

Conclusions: There are individual differences in the distribution of esophageal flora for ESCC. The diversity and distribution of esophageal tissues are reduced and disordered compared to normal esophageal tissues. There are no correlations between distinct parts and stages of ESCC and esophageal flora, while morphological types and lymph node metastasis can affect the structure of esophageal flora.

Keywords: Esophagus flora; esophageal squamous cell carcinoma (ESCC); distribution characteristics; pathological characteristics; correlation

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Introduction

Epidemiological characteristics of esophageal cancer: Esophageal cancer is one of the most common malignant tumors in the digestive system in China, with its morbidity ranking first in the world. The annual rate of increase and mortality rate of esophageal cancer in China accounts for 50% of the word (1). The vast majority of patients with esophageal cancer have no apparent clinical symptoms in the early stage and low detection rate of gastroscopy, which causes most patients to miss the best treatment opportunities, which affects the prognosis of patients. Therefore, it is a crucial point to improve the early diagnosis rate of esophageal cancer. At present, the specific pathogenic factors and mechanisms of esophageal cancer are not yet apparent, but reports are showing that esophageal cancer is related to many factors, including chronic stimulation of the esophagus by smoking, hot food, and pickling, environmental and genetic factors (2). The pathology of esophageal cancer is a multi-stage, multi-link, and multi-factor step-by-step process. The occurrence of chronic inflammation is inevitable. Previous data have reported that nearly one-fourth of malignant tumors are associated with chronic inflammation, of which the infection of biological pathogens are the leading causes (3).

Progress in the study of esophageal flora: There are a large number of symbiotic microorganisms in the human ecosystem, and their cell number is about 10 times that of human germ cells and non-germ cells, which is said to be the second largest genome of the human body. In the past, based on the limitation of traditional culture methods, the esophagus was considered to be sterile, or only a small portion of transient bacteria from the oral cavity or gastroesophageal reflux (4). Because using the traditional culture methods, it not only shows poor accuracy, taking time and effort, but also most bacteria cannot be cultivated, isolated and purified. However, more and more studies have found that there is complex resident flora on the surface of esophageal mucosa, which cannot be removed by simple flushing (5,6). In recent years, with the continuous advancement of molecular biology technology and people's understanding of the role of pathogenic microorganisms in tumorigenesis, many non-cultivation methods (such as 16S rRNA gene sequencing method) have been widely used in the field of microbiology (7,8), and the research on the correlation between the digestive tract flora, specific pathogenic bacteria and the occurrence of digestive tract tumors has also received increasing attention. 16S rRNA is the DNA sequence coding rRNA on the bacterial chromosome, which exists in the chromosomes of many prokaryotes (bacteria, chlamydia, mycoplasma, rickettsia, spirochetes, actinomycetes and other microorganisms), while not in non-prokaryotic organisms such as fungi and viruses (9). The bacterial 16S rRNA gene sequence is composed of two regions, which are conserved regions and variable regions. The conserved regions can reveal the biological relationship between biological species, and the variable regions can reflect biological specific nucleic acid sequences, which is the molecular basis for bacterial identification. At present, 16S rRNA gene sequencing of almost all common bacteria has been completed, and 16S rRNA is ubiquitous in bacteria, suitable for analysis of all bacteria. Therefore, we can use the 16S rRNA gene sequencing method to analyze the esophageal mucosa flora in patients with esophageal cancer and identify the bacterial species.

Relationship between esophageal cancer and esophageal flora: The human body is the largest reservoir of bacteria, in which the flora in the digestive tract and the human body exists mutually. Repeated episodes of chronic reflux esophagitis will increase the risk of esophageal cancer. Pei and others have shown that there are a large number of complex resident flora in the esophagus mucosa of patients with reflux esophagitis (10). Yu *et al*. reported that compared with normal and adjacent tissues, the incidence rate of Helicobacter pylori (Hp) infection in cancer tissues of patients with esophageal cancer is significantly higher (11), which is like previous studies (12). Another study found that Clostridium and Erysipelothrix (Firmicutes) and streptococci may be closely related to the occurrence of esophageal cancer (13). The above studies have suggested that the incidence rate of esophageal cancer may be related to changes in mucosal flora. Therefore, this study aims to analyze the changes and distribution characteristics of esophageal cancer flora, compare the differences between different pathological characteristics, and explore the relationship between esophageal florae. Furthermore, we aim to measure the occurrence and progression of esophageal cancer and supply new directions and references for early screening and prevention research for esophageal cancer. We present the following article in accordance with the STROBE reporting checklist (available at http://dx.doi.org/10.21037/tcr-20-1954).

Methods

Research object

Fifty-four patients with esophageal cancer who were admitted to our hospital from June 2018 to January 2020 were selected as the research objects, including 24 males and 30 females, aged 28 to 75 years, with an average age (58.79±1.20) years. Inclusion criteria were as follows: (I) all patients underwent radical resection of esophageal carcinoma in our hospital and were confirmed as ESCC by pathologic diagnosis and gastroscopy (14); (II) no chemotherapy was performed before surgery; (III) no treatment with antibiotics or biological agents before surgery were used; (IV) The studies were conducted in accordance with the Declaration of Helsinki and were approved by the hospital ethics committee; (V) All patients and their families signed informed consent to participate in this study. Exclusion criteria were as follows: (I) patients with contraindications to radical resection of esophageal carcinoma; (II) Secondary malignant cases of esophageal squamous cell carcinoma; (III) Recurrence of esophageal squamous cell carcinoma after treatment; (IV) patients who underwent esophageal surgery for other reasons; (V) patients with other malignant tumors; (VI) incomplete information or lack of accuracy.

General survey and clinical data collection

For a general survey, patients were investigated using a questionnaire prepared by researchers in a coordinated manner. The survey contents included gender, age, telephone numbers, residence, marital status, educational level, lifestyle (drinking, smoking, tea drinking), medical history, family history, eating habits, mood, and other information. The survey was performed as a face-to-face interview between the investigator and the patients, and after all the patients informed consent to this study and signing the informed consent form, we did the investigation and the relative examination. Clinical data collection included the course of the disease, location of the lesion, different morphological types, and whether with lymph node metastasis.

Collection of tissue specimens

Fifty esophageal cancer tissues from the patients were cut (one per person) and four normal esophageal tissues (surgical stump at the edge of the lesion >5 cm) during radical esophageal cancer surgery. All specimens were at once placed into an autoclaved cryopreservation tube to froze in liquid nitrogen within 1 hour of cutting, and then store in –80 ℃ refrigerator for testing. The medical scissors used for cutting tissue samples were all sterile scissors in the operating room, and the scissors used for cutting were replaced for each site.

Main reagents and instruments used in testing

For analysis, the following instruments were used: NanoDrop® ND-2000 (NanoDrop, UK); PCR instrument (Applied Biosystems, USA); Nucleic acid electrophoresis instrument (Bio-Rad, USA); Desktop refrigerated high-speed centrifuge (Z323K) (HERMLE, Germany); The ultra-low temperature refrigerator of –80 ℃ (Thermo Fisher, USA). And agents used were as follows: TIANamp Genomic DNA Kit (TIANGEN, Beijing, China); Qubit2.0 DNA detection kit (Invitrogen, Carlsbad, USA); SanPrep column DNA recovery kit (Shanghai Biological Engineering, China).

Extraction and detection of total genomic DNA from tissue specimens

TIANamp Genomic DNA Kit was used to extract the total DNA of all tissues. The concentration and purification of total DNA extracted was measured by NanoDrop ND-2000 nucleic acid quantifier. The Optical Density (OD value) was the ratio of absorbance at 260 and 280 nm to determine the purity of the extracted DNA, and the result of OD values of the extracted total DNA was between 1.8 and 2.0. Then the purity was confirmed by 1% agarose gel electrophoresis detection with the voltage of 240–250 V, running for 30 min. The UVI gel imaging system was used for photo recording. If the results show that no other bands or smears appear, showing that the DNA fragments are of excellent purity without significant degradation. All extracted DNA was stored in a refrigerator at –20 ℃ until use.

PCR amplification of bacterial 16S rRNA gene

The amplification of the 16S rRNA gene was conducted using genomic DNA as a template, with bacterial universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (ACGGCTACCTTGTTACGACTT). The PCR reaction system (20 μL) included: Premix 10 μL (TAKARA, Japan), upstream and downstream primers 27F and 1492R

(10 μmol/L) of 1 μL for each primer, template DNA of 1–2 μL (quantitatively equivalent to 200 ng by nucleic acid quantifier), adding double distilled water to 20 μL. The negative control was the same as the sample except that no template DNA was added.

PCR reaction program: pre-denaturation at 94 ℃ for 5 min, then reaction for a total of 25 cycles (denaturation at 94 ℃ for 30 s, annealing at 56 ℃ for 30 s, 72 ℃ extension for 1 minute), and finally extension for 8 minutes at 72 ℃. PCR products were detected by running the 2% agarose gel electrophoresis at voltage 110 V for 46 min, and the gel was photographed by UVI gel imaging system. The products of the PCR reactions were then stored in a refrigerator at –20 ℃ for use.

High-throughput sequencing

Qubit 2.0 DNA detection kit was used to quantify the genomic DNA to determine the amount of DNA that should be added to the PCR reaction. The primers were performed for PCR reaction after being integrated with V3-V4 universal primers of MiSeq sequencing platform as 341 F: CCCTACACGACGCTCTTCCGATCTG (barcode) CCTACGGGNGGCWGCAG; 805 primers: GACTGGAGTTCCTTGGCACCCGAGAATTCCA (barcode) GACTACHVGGGTATCTAATCC.

Agarose recovery

The DNA was recovered and purified using the agarose recovery kit from Shanghai Biological Engineering after running the agarose gel (cat: SK8131). After the electrophoresis was completed, the gel at the target position of the high-brightness band was cut under the irradiation of the ultraviolet lamp of the gel image system and put in a 1.5 mL tube for weighing. Then add the binding buffer (weight: volume =1:1) to the tube and incubate at 56 ℃ until the gel was completely melted. Take 700 μL of the above solution into a 2 mL collection tube with Hibind DNA column, at room temperature 10,000 g, centrifuge for 1 min, and discard the waste solution. Take 300 μL of binding buffer into a 2 mL collection tube with Hibind DNA column, centrifuge at 10,000 g, at room temperature for 1 min, and discard the waste solution. Add 700 μL of SPW Wash Buffer (diluted with absolute ethanol), centrifuge at 10,000 g at room temperature for 1min, and discard the liquid, and then centrifuge the empty tube with Hibind DNA column at 13,000 g, at room temperature for 2 min to remove absolute ethanol. Place the Hibind DNA column in a new 1.5 mL centrifuge tube, add 30 μL of Elution buffer (preheated to 60 ℃), centrifuge at 13,000 g, at room temperature for 1 min. The Qubit 2.0 DNA detection kit was used to accurately quantify the concentration of recovered DNA, followed by 1:1 equal mixing and parallel sequencing. In the end, 10 ng of DNA was taken from each sample, and the concentration of sequencing on the computer was 20 pmol.

Miseq sequencing

Illumina's Miseq platform was used for bidirectional sequencing, and the raw sequence data needed to be subjected to quality control (QC). The OTU information was used to analyze the diversity and relative abundance of the esophageal microbial community. The Mothur software was used to calculate the diversity indexes of 5 species, including Chao, Shannon, ACE, Simpson, and Coverage, and to analyze the species diversity of samples. The Chao index and ACE index were two commonly used indicators to estimate the total number of species. The Shannon index was used to measure the heterogeneity of the bacterial community. The calculation formula is $H_{Shannon} = -\sum Pi * lnPi$ (15), Pi is the ratio of the number of species in each population to the total species in the sample. Simpson index is an indicator to measure the diversity of flora. The formula is $D_{Simpson} = \sum ni\ (ni-1)/[N\ (N-1)]$ (16), where *ni* is the number of OTUs with i sequences, and N represents the total number of sequences. For both the Shannon index and Simpson index, the larger the value, the higher the colony diversity. The coverage index refers to the coverage rate of each sample library. The calculation formula is: $C = [1 – n/N]$ (17), *n* means the number of OTUs containing only one sequence, and N is the total number of sequences in the sampling. The representative sequences were blasted with the Greengene core 16S rRNA gene sequence for strain identification, and the Unifrac algorithm in Mothur software was used to calculate sample distance, sample clustering, and sample principal component analysis (PCA).

Research content

According to the results of 16S rRNA gene sequencing, the distribution of esophageal flora in esophageal cancer mucosal tissue was recorded and calculated, and the distribution of esophageal flora in the mucosal tissues of

Table 1 Clinical data of 54 patients with ESCC

Clinical data	Cases (n=54) (%)
The course of disease (year)	
<2	22 (40.74)
≥2	32 (59.26)
Lesion sites	
The upper thoracic portion	8 (14.81)
The mid-thoracic portion	22 (40.74)
The lower thoracic portion	24 (44.44)
Morphological type	
Medullary type	26 (48.15)
Ulcerative type	18 (33.33)
Other types	10 (18.52)
Tumor TNM stage	
T1–T2	20 (37.04)
T3–T4	34 (62.96)
Lymph node metastasis	
Yes	14 (25.93)
No	40 (74.07)

ESCC, esophageal squamous cell carcinoma.

esophageal cancer was compared among different tissue samples, different pathological sites, different morphological types, and with or without lymph node metastasis

Statistical analysis

The data in this study were all statistically analyzed using SPS 24.0 software. The measurement data following the normal distribution were described as mean ± standard deviation (SD), and analyzed using *t*-test or analysis of variance; the count data were expressed as the rate or composition ratio, and was analyzed by χ^2-test; the rank-sum test was used for the analysis of non-normal distribution data. A difference was considered significant at P<0.05.

Results

Clinical data of 54 patients with ESCC

There was a more frequent occurrence of ESCC that was more common in the middle and lower thorax in 54 patients. The morphology was medulla type, and most of the tumor stages were T3 to T4. Lymph node metastasis was rare to see (*Table 1*).

Distribution of esophageal flora in ESCC tissues and normal tissues

The data and detection results showed that in the ESCC mucosal tissues, at the genus level, Proteus, Firmicutes, Bacteroides, and Fusobacterium were the main dominant bacteria in esophageal cancer tissues, and their abundance is high in the tissues. From the perspective of the genus level, the abundance of Prevotella, Clostridia, Streptococcus, Delftia, Klebsiella, Serratia, and some unclassified florae were high and belonged to the dominant species in esophageal cancer tissues. In normal healthy esophageal mucosal tissues, at the genus level, Prevotella, unclassified bacteria, and Streptococcus were the dominant bacteria, and Klebsiella, Shewanella, Peptostreptococcus, Porphyromonas, Lactobacillus, Delftia, Serratia, Clostridium, Gemella, Parvimonas, Prevotella, and Photobacterium were also common to see.

Comparison of esophageal flora diversity in different tissues

According to the sequencing results, there was no significant difference between the Simpson index of esophageal cancer mucosa tissue and normal esophageal mucosa tissue (0.017±0.002 *vs.* 0.016±0.002), (t=1.305, P=0.198), while Shannon index of esophageal cancer mucosa tissue was significantly lower than normal Mucosal tissues (5.632±0.010 *vs.* 6.381±0.013), (t=187.035, P<0.001), which suggested that the community heterogeneity of the esophageal flora was different between esophageal cancer tissues and normal esophageal mucosa tissues, but with no significant difference in the diversity.

Comparison of esophageal flora abundance in different tissues

There was no significant difference in bacteria abundance between ESCC tissues and normal cancer tissues (P>0.05), and they were more in common with Firmicutes, Proteobacteria, Bacteroides, and Clostridium. The abundance of Firmicutes and Proteobacteria was higher than others (*Table 2*).

Table 2 Comparison of abundance of esophageal flora in different tissues

Esophageal flora	n	Abundance	t	P
Firmicutes			0.979	0.365
Squamous carcinoma tissues	4	8,418.262±569.301		
Normal esophageal tissue	4	8,026.342±562.484		
Proteobacteria			1.165	0.288
Squamous carcinoma tissues	4	8,828.360±609.101		
Normal esophageal tissue	4	9,342.267±638.302		
Bacteroidetes			0.212	0.839
Squamous carcinoma tissues	4	2,046.646±624.368		
Normal esophageal tissue	4	1,954.303±608.971		
Fusobacteria			0.097	0.926
Squamous carcinoma tissues	4	2,136.468±541.301		
Normal esophageal tissue	4	2,098.633±560.307		
Actinobacteria			0.332	0.751
Squamous carcinoma tissues	4	564.305±103.468		
Normal esophageal tissue	4	589.689±112.321		
Spirochaetae			0.485	0.645
Squamous carcinoma tissues	4	158.620±48.427		
Normal esophageal tissue	4	142.366±46.481		

Table 3 Comparison of the diversity of esophageal flora in ESCC tissues

Lesion sites	n	Shannon index	Simpson index
The upper thoracic portion	8	5.306±0.008	0.016±0.001
The mid-thoracic portion	22	5.310±0.009	0.017±0.001
The lower thoracic portion	24	5.312±0.007	0.017±0.001
F	–	1.710	3.240
P	–	0.192	0.058

ESCC, esophageal squamous cell carcinoma.

Comparison of esophageal flora diversity in ESCC tissues at different sites

There was no significant difference in bacterial diversity in ESCC tissues at different sites (P>0.05, *Table 3*).

Comparison of esophageal flora abundance in ESCC tissue at different sites

There was no significant difference in the abundance of distinct flora in ESCC tissue at different locations (P>0.05). The abundance of Firmicutes, Proteobacteria and Bacteroides were significantly higher than Fusobacterium, Actinobacteria and Spirochaetae, and Spirochaetae were with the lowest abundance (*Table 4*).

Comparison of esophageal flora diversity in different morphological ESCC tissues

There was no significant difference in esophageal flora

Table 4 Comparison of abundance of esophageal flora in ESCC tissues in different sites

Esophageal flora	n	Abundance	F	P
Firmicutes			2.190	0.123
The upper thoracic portion	8	6,023.364±428.312		
The mid-thoracic portion	22	6,248.647±436.541		
The lower thoracic portion	24	5,997.640±408.946		
Proteobacteria			1.470	0.239
The upper thoracic portion	8	4,484.632±368.749		
The mid-thoracic portion	22	4,698.621±379.634		
The lower thoracic portion	24	4,748.987±380.548		
Bacteroidetes			1.490	0.235
The upper thoracic portion	8	2,497.587±287.642		
The mid-thoracic portion	22	2,794.158±287.643		
The lower thoracic portion	24	2,647.962±289.684		
Fusobacteria			0.360	0.700
The upper thoracic portion	8	897.640±298.638		
The mid-thoracic portion	22	976.546±301.364		
The lower thoracic portion	24	909.897±296.894		
Actinobacteria			2.580	0.085
The upper thoracic portion	8	259.365±68.264		
The mid-thoracic portion	22	226.367±26.846		
The lower thoracic portion	24	236.547±25.346		
Spirochaetae			1.730	0.188
The upper thoracic portion	8	236.634±89.346		
The mid-thoracic portion	22	186.342±59.634		
The lower thoracic portion	24	207.320±66.576		

ESCC, esophageal squamous cell carcinoma.

diversity between different morphological ESCC tissues (P>0.05, *Table 5*).

Comparison of esophageal flora abundance in different morphological ESCC tissues

There was no significant difference in the abundance of Firmicutes, Proteobacteria, Bacteroides and Fusobacteria in different morphological ESCC tissues (P>0.05), while there were differences in Actinobacteria and Spirochaetae. The abundance of Spirochaetae of ulcerative type was significantly higher than Medullary type, while the abundance of Actinobacteria of both Medullary type and ulcerative type were significantly lower than other types (P<0.05). But there was no significant difference in the abundance of Actinobacteria of these two types (P>0.05, *Table 6*).

Comparison of esophageal flora diversity in different tumor stages and lymph node metastasis in esophageal cancer mucosal tissues

There was no significant difference in esophageal flora diversity in different tumor stages and different lymph

Table 5 Comparison of the diversity of esophageal flora in ESCC mucosa with different morphologies

Morphological type	n	Shannon index	Simpson index
Medullary type	26	5.218±0.011	0.019±0.002
Ulcerative type	18	5.220±0.010	0.018±0.001
Other types	10	5.2194±0.011	0.018±0.001
F	–	0.120	2.41
P	–	0.890	0.099

ESCC, esophageal squamous cell carcinoma.

Table 6 Comparison of abundance of esophageal flora in mucosal tissues of ESCC with different morphologies

Esophageal flora	n	Abundance	F	P
Firmicutes			1.88	1.623
Medullary type	26	6,258.679±598.362		
Ulcerative type	18	6,420.368±592.036		
Other types	10	6,676.320±603.264		
Proteobacteria			1.050	0.358
Medullary type	26	6,036.536±234.026		
Ulcerative type	18	5,899.634±226.304		
Other types	10	5,936.248±230.203		
Bacteroidetes			1.650	0.201
Medullary type	26	2,548.624±209.362		
Ulcerative type	18	2,830.364±214.230		
Other types	10	2,730.260±220.302		
Fusobacteria			2.500	0.092
Medullary type	26	593.364±89.306		
Ulcerative type	18	665.307±86.348		
Other types	10	623.579±85.206		
Actinobacteria			39.840	<0.001
Medullary type	26	328.964±103.264*		
Ulcerative type	18	219.321±89.346*[&]		
Other types	10	510.387±129.864		
Spirochaetae			374.060	<0.001
Medullary type	26	58.697±19.364[#]		
Ulcerative type	18	610.149±89.306		
Other types	10	186.459±30.264		

[&], P>0.05, compared with medullary type; [#], P<0.05, compared with ulcerative type; *, P<0.05, compared with other types. ESCC, esophageal squamous cell carcinoma.

Table 7 Comparison of the diversity of esophageal flora in esophageal cancer mucosa tissues with different tumor stages and whether with lymph node metastasis

Clinical data	n	Shannon index	Simpson index
Tumor stage			
T1–T2	20	5.020±0.008	0.016±0.001
T3–T4	34	5.028±0.008	0.017±0.001
t	–	0.887	0.862
P	–	0.379	0.381
Lymph node metastasis			
Yes	14	5.028±0.010	0.017±0.001
No	40	5.026±0.010	0.018±0.001
t	–	0.710	0.802
P	–	0.481	0.336

node metastasis status of esophageal cancer mucosal tissues (P>0.05), suggesting the similarity and consistency of esophageal flora in esophageal cancer mucosal tissues of different stages and with or without lymph node metastasis (*Table 7*).

Comparison of esophageal flora abundance in esophageal cancer mucosal tissues with different tumor stages

There was no significant difference in esophageal flora abundance in different tumor stages of esophageal cancer mucosa tissues (P>0.05, *Table 8*).

Comparison of esophageal flora abundance in esophageal cancer mucosa tissues with or without lymph node metastases

There was no significant difference in the abundance of Firmicutes, Fusobacteria, Actinobacteria and Spirochaetae in different lymph node metastatic esophageal cancer mucosa tissues (P>0.05). However, the abundance of proteobacteria in mucosa tissues with lymph node metastasis was significantly decreased, while the abundance of Bacteroides was significantly increased, compared with that without lymph node metastasis (P<0.05, *Table 9*).

Discussion

In recent years, the incidence rate of malignant tumors in China is caused by several factors such as lifestyle and dietary structure, which has shown a rising trend at an annual growth rate of about 5% (18). It is reported that in the 2008 new cancer statistics, 16% of cancers are related to infections (19). Compared with developed countries, the incidence of cancers caused by repeated infections is higher in developing countries (20), and the most important causes of infection are bacteria and viruses (21). However, reports on the correlation between specific flora and esophageal cancer are rare (22). In this study, we analyzed the clinical and pathological data of 54 patients with ESCC, and the distribution of esophageal flora in ESCC tissues by Miseq high-throughput sequencing technology and bioinformatics, as well as the correlation between esophageal flora and the clinicopathologic features of ESCC, to help clinical prevention and treatment of esophageal cancer.

The results demonstrated that at the genus level, Proteus, Firmicutes, Bacteroides, and Fusobacterium are the main dominant bacteria in esophageal cancer tissues with high abundance in the tissues. From the perspective of genus level, the abundance of Platts, Clostridium, Streptococcus, Delft, Klebsiella, Serratia and some unclassified florae were high and belonged to the dominant bacteria in esophageal cancer tissues. This result shows the basic distribution of esophageal flora in ESCC, which has led us to a preliminary understanding of esophageal flora. However, the deep correlation needs further study. Further analysis showed that there was no significant difference in the bacterial abundance between ESCC tissues and normal cancer tissues, and they were more in common with Firmicutes, Proteobacteria, Bacteroides, and Clostridium.

Table 8 Comparison of abundance of esophageal flora in esophageal carcinoma mucosa tissues of different tumor stages

Esophageal flora	n	Abundance	Z	P
Firmicutes			−1.292	0.202
T1–T2	20	7,268.640±456.320		
T3–T4	34	7,103.156±453.26		
Proteobacteria			−1.837	0.072
T1–T2	20	7,935.631±402.302		
T3–T4	34	7,725.982±406.589		
Bacteroidetes			−1.441	0.156
T1–T2	20	5,063.245±235.126		
T3–T4	34	4,969.346±228.965		
Fusobacteria			−01.864	.068
T1–T2	20	2,536.047±267.102		
T3–T4	34	2,398.647±258.364		
Actinobacteria			−1.711	0.093
T1–T2	20	768.366±86.310		
T3–T4	34	698.394±80.864		
Spirochaetae			−0.498	0.619
T1–T2	20	268.964±20.647		
T3–T4	34	266.064±20.364		

The abundance of Firmicutes, Proteobacteria, was higher than the others. However, ESCC has a lower diversity of bacteria, which suggests that the occurrence of ESCC might cause the focal change of esophageal flora. The reduction in esophageal flora diversity means a reduction in the number of species or a reduction in the number and abundance of strains in a single species (23). The occurrence of the above-mentioned conditions can lead to disorders of the esophageal flora system, which in turn causes the weakness of the esophagus' ability to inhibit pathogenic bacteria, leading to infection inflammation and eventually esophageal cancer. The reasons for this result may be related to dietary differences, lifestyle habits, living environment and health status among individuals. The occurrence of the above phenomenon can lead to disorders of the esophagus flora system, reducing the ability of the esophagus to inhibit pathogenic bacteria, leading to infection and inflammation, and then esophageal cancer. In previous related studies (24), Prevotella, Streptococcus, and Veillonella were the main colonies in the healthy normal esophageal mucosal tissues, and the results showed that Prevotella, unclassified bacteria and Streptococcus were the main dominant bacteria in healthy normal esophageal tissues, which was consistent with the results in previous research.

Also, the results showed that there was no significant difference in the diversity of bacterial flora in ESCC tissues at different sites, different morphology, different staging, and different lymph node metastasis. There was no significant difference in the bacterial abundance in the mucosal tissues of ESCC at different sites. However, the abundance of Firmicutes, Proteobacteria, and Bacteroides was significantly higher than Fusobacteria, Actinobacteria, and Spirochaetae, with the lowest abundance of Spirochaetae, which suggests that for the study of esophageal cancer flora, there are no special requirements on the location of the specimen tissue, and any part of the cancerous tissue can be used to analyze the changes of the esophageal flora. This study also shows that the abundance of Proteobacteria in the mucosal tissue with lymph node metastasis was significantly reduced. In contrast, the abundance of Bacteroides was significantly increased, showing that the dominant species were reduced, and the

Table 9 Comparison of abundance of esophageal flora in esophageal carcinoma mucosa tissues with different lymph node metastases

Esophageal flora	n	Abundance	Z	P
Firmicutes			−0.436	0.665
Lymph node metastasis	14	6,086.964±228.654		
No lymph node metastasis	40	6,056.246±226.348		
Proteobacteria			−24.109	<0.001
Lymph node metastasis	14	5,036.584±385.329		
No lymph node metastasis	40	8,264.326±445.368		
Bacteroidetes			−23.097	<0.001
Lymph node metastasis	14	3,698.324±286.346		
No lymph node metastasis	40	1,986.987±220.397		
Fusobacteria			−0.979	0.332
Lymph node metastasis	14	1,286.346±293.647		
No lymph node metastasis	40	1,198.698±286.576		
Actinobacteria			−1.691	0.097
Lymph node metastasis	14	542.367±50.864		
No lymph node metastasis	40	516.667±48.306		
Spirochaetae			−1.828	0.073
Lymph node metastasis	14	245.967±29.303		
No lymph node metastasis	40	229.634±28.598		

secondary flora was increased, suggesting that with the development of the disease, the changes in the esophagus flora may also relate to tumor metastasis. There were differences in Actinobacteria and Spirochaetae in different morphological ESCC tissues. For example, the abundance of Spirochaetae of ulcerative type was significantly higher than Medullary type, and the abundance of Actinobacteria of both these two types was significantly lower than other types, suggesting that close monitoring and follow-up of patients with esophageal ulcers may help early screening for esophageal cancer and preventing the occurrence of esophageal cancer. However, whether the occurrence of ESCC is because the ulceration induced by the reduction of Actinobacteria needs to be further studied through increasing the sample content and excluding other effects.

In summary, there are individual differences in the distribution of esophageal flora in ESCC, of which the diversity and distribution of esophageal tissue are reduced and disordered compared to healthy normal esophageal tissue. There is no correlation between different sites and stages of ESCC and esophageal flora, while morphology and lymph node metastasis can affect the structure of esophageal flora. And in recent years, with the deepening of the epidemiological research on digestive tract tumors, the relationship between microorganisms and digestive tract tumors has gradually been valued. At present, there are no specific drugs for the change of esophageal cancer flora, but with the continuous development and improvement of molecular biology technology and related experimental methods, and the continuous deepening of people's research on the role of bacteria in the pathogenesis of digestive tract tumors, it will be possible to treat esophageal cancer by targeting esophageal flora. At the same time, it may be used as a biomarker to develop a simple and easy new method for early detection of esophageal cancer, which provides a brand-new method for the diagnosis and treatment of esophageal cancer.

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References

1. Li H, Fang W, Yu Z, et al. Chinese expert consensus on mediastinal lymph node dissection in esophagectomy for esophageal cancer (2017 edition). J Thorac Dis 2018;10:2481-9.
2. Hong L, Zhang Y, Liu Z. Neuroendocrine carcinoma of esophageal and gastric cardia: clinicopathologic and immunohistochemistry study of 80 cases. Oncotarget 2017;9:10754-64.
3. Lau KW, Zeng H, Liang H, et al. Bioinformatics-based identification of differentiated expressed microRNA in esophageal squamous cell carcinoma. Transl Cancer Res 2018;7:1366-75.
4. Gagliardi D, Makihara S, Corsi PR, et al. Microbial flora of the normal esophagus. Dis Esophagus 1998;11:248-50.
5. Cassone A, Cauda R. Candida and candidiasis in HIV-infected patients: where Commensalism, opportunistic behavior and frank pathogenicity lose their borders. AIDS 2012;26:1457-72.
6. Ye Y, Dandan M, Jianxin Z. Expression of FAM134B in hepatocellular carcinoma and its relationship with clinicopathological features. Acta Universitatis Medicinalis Anhui 2019;14:398-9.
7. Jazeron JF, Barbe C, Frobert E, et al. Virological diagnosis of herpes simplex virus1 esophagitis by quantitative real-time PCR assay. J Clin Microbiol 2012;50:948-52.
8. Shibuya K, Coulson WF, Wollman JS, et al. Histopathology of cryptococcosis and other fungal infections in patients with acquired immunodeficiency syndrome. Int J Infect Dis 2001;5:78-85.
9. Li P, Ma YJ, Zhao Y. Application of 16S rRNA, 23S rRNA and 16S~23S rRNA genes in the isolation and identification of bacteria. Modern Journal of Animal Husbandry and Veterinary Medicine 2008;8:49-52.
10. Pei Z, Yang L, Peek RM, et al. Bacterial biota in reflux esophagitis and Barrett's Esophagus. World J Gastroenterol 2005;11:7277-83.
11. Yu DH, Cheng ZN, Jia JH, et al. Relation between Spirochaetae pylori L-form Infection and tumor angiogenesis in human esophageal carcinoma. Chinese Journal of Oncology 2003;25:51-4.
12. Shao D, Xie SH, Wei WQ. The esophageal microbiota in health and disease. Chinese Journal of Preventive Medicine 2017;51:443.
13. Norder Grusell E, Dahlén G, Ruth M, et al. The cultivable bacterial flora of the esophagus in subjects with esophagitis. Scand J Gastroenterol 2018;53:650-6.
14. Wickborn C, Klein-Szanto AJ, Schlag PM, et al. Correlation of visinin-like-protein-1 expression with clinicopathological features in squamous cell carcinoma of the esophagus. Mol Carcinog 2006;45:572-81.
15. Fujii Y, Fujii K, Iwata S,, et al. Abnormal intracellular distribution of NFAT1 in T lymphocytes from patients with systemic lupus erythematosus and characteristic clinical features. Clin Immunol 2006;119:297-306.
16. Liu AQ, Chen W. Advances in esophageal microbiota and esophageal related diseases. Zhonghua Zhong Liu Za Zhi 2019;41:561-4.
17. Zhang GJ, Zhao J, Jiang ML, et al. ING5 inhibits cell proliferation and invasion in esophageal squamous cell carcinoma through regulation of the Akt/NF-κB/MMP-9 signaling pathway. Biochem Biophys Res Commun

2018;496:387-93.
18. Yu Q, Liu Y, Wen C, et al. MicroRNA-1 inhibits tumorigenicity of esophageal squamous cell carcinoma and enhances sensitivity to gefitinib. Oncol Lett 2018;15:963-71.
19. Kunizaki M, Hamasaki K, Wakata K, et al. Clinical Value of Serum p53 Antibody in the Diagnosis and Prognosis of Esophageal Squamous Cell Carcinoma. Anticancer Res 2018;38:1807-13.
20. Cui Q, Wu C, Lin D. Genomic alterations and precise medicine of esophageal squamous cell carcinoma. Journal of Bio-X Research 2018;1:7-11.
21. Kai J, Wang Y, Xiong F, et al. Genetic and pharmacological inhibition of eIF4E effectively targets esophageal cancer cells and augments 5-FU's efficacy. J Thorac Dis 2018;10:3983-91.
22. Zhou H, Liu Y, Zhu R, et al. OTUB1 promotes esophageal squamous cell carcinoma metastasis through modulating Snail stability. Oncogene 2018;37:3356-68.
23. Wang W, Wei C, Li P, et al. Integrative analysis of mRNA and lncRNA profiles identified pathogenetic lncRNAs in esophageal squamous cell carcinoma. Gene 2018;661:169-75.
24. Bao Y, Zhang S, Guo Y, et al. Stromal expression of JNK1 and VDR is associated with the prognosis of esophageal squamous cell carcinoma. Clin Transl Oncol 2018;20:1185-95.

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