Exploring Gut Microbiota Characteristics in Esophageal Squamous Cell Carcinoma Patients by 16SrDNA Sequencing: Postprint

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Abstract

Objective To explore the fundamental characteristics of gut microbiota in patients with esophageal squamous cell carcinoma.

Methods Thirty-five treatment-naive patients with esophageal squamous cell carcinoma were enrolled as the esophageal cancer group (EC group), and 35 healthy volunteers were enrolled as the control group (DZ group). Fecal specimens were collected from both cohorts, and gut microbiota was profiled using 16S rDNA sequencing. Alpha diversity, Beta diversity, and differential microbial taxa were analyzed based on species annotation results.

Results No significant differences were observed in Alpha diversity indices, including Shannon, Simpson, Chao1, and ACE, between the two groups ($P<0.05$). However, principal coordinate analysis (PCoA) of Beta diversity revealed distinct clustering patterns, indicating differential microbial community structures. At the genus level, t-test analysis of differential species demonstrated decreased abundances of Faecalibacterium, Roseburia, and Citrobacter, and increased abundances of Romboutsia, Ruminococcus_{torques}_{group}, Intestinibacter, and Turicibacter in the EC group compared with the DZ group ($P<0.05$). LEfSe analysis showed that at both genus and species levels, compared with the DZ group, g-faeculibacterium and s-faecalibacterium-prausnitzii were significantly depleted, while g-Romboutsia and s-Rombutsia-ilealis were significantly enriched in the EC group ($P<0.05$).

Conclusion Patients with esophageal squamous cell carcinoma harbor significantly altered gut microbiota, with Faecalibacterium, Faecalibacterium prausnitzii, Romboutsia, and Rombutsia-ilealis representing potentially disease-specific taxa that may be implicated in esophageal carcinogenesis.

Full Text

Exploring the Characteristics of Gut Microbiota in Patients with Esophageal Squamous Cell Carcinoma Based on 16S rDNA Sequencing

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Abstract

Objective: To investigate the fundamental characteristics of gut microbiota in patients with esophageal squamous cell carcinoma (ESCC).

Methods: Thirty-five treatment-naive ESCC patients were enrolled as the esophageal cancer group (EC group), and 35 healthy volunteers served as the control group (DZ group). Fecal samples were collected from both cohorts and analyzed using 16S rDNA sequencing. Based on the species annotation results, we analyzed alpha diversity, beta diversity, and differential microbial taxa between the two groups.

Results: No significant differences were observed between the two groups in alpha diversity indices, including Shannon, Simpson, Chao1, and ACE (P>0.05). However, principal coordinate analysis (PCoA) of beta diversity revealed distinct separation between the two cohorts, indicating differences in microbial community structure. T-test analysis of differential species showed that at the genus level, the EC group exhibited decreased abundance of *Faecalibacterium*, *Roseburia*, and *Citrobacter*, while *Romboutsia*, *Ruminococcus_{torques}_{group}, Intestinibacter, *and* Turicibacter* were significantly enriched compared to the DZ group (P<0.05). LEfSe analysis further demonstrated that at both genus and species levels, *Faecalibacterium* and *Faecalibacterium prausnitzii* were significantly reduced, whereas *Romboutsia* and *Romboutsia ilealis* were markedly elevated in the EC group (P<0.05).

Conclusion: ESCC patients exhibit significant alterations in gut microbiota composition. *Faecalibacterium*, *Faecalibacterium prausnitzii*, *Romboutsia*, and

Romboutsia ilealis may represent specific biomarkers associated with esophageal cancer and are closely related to its pathogenesis.

Keywords: Esophageal squamous cell carcinoma; intestinal flora; 16S rDNA sequencing

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Introduction

Esophageal cancer demonstrates marked geographic variation, with recent statistics indicating that both incidence and mortality in China account for over 50% of global cases—more than double the world average. Cixian and Shexian counties in Hebei Province represent high-incidence regions in China. ESCC comprises over 90% of esophageal cancer cases in China, and while early-stage patients have favorable outcomes following radical surgery, most present at advanced stages, resulting in a five-year survival rate of only approximately 20%. Therefore, early diagnosis and treatment are critically important. In our clinical practice, we have observed that most esophageal cancer patients experience dry stools, with some having bowel movements only once every ten days or more, suggesting that such intestinal environments must harbor altered microbial communities. Numerous studies have confirmed that gut microbiota is closely associated with various tumors, including colorectal cancer, gastric cancer, and hepatocellular carcinoma, and can serve as a reference indicator for diagnosis, treatment, and prognosis. However, research on gut microbiota characteristics in esophageal cancer patients remains limited. This study aims to explore the gut microbiota profile in ESCC patients, with the goal of identifying specific differential bacteria to provide evidence-based support for early diagnosis and treatment.

Methods

1.1 Study Population This study enrolled 35 ESCC patients from the Fourth Hospital of Hebei Medical University between April 2022 and August 2022 as the EC group. Concurrently, 35 healthy volunteers undergoing physical examinations at the same hospital were recruited as the DZ group. The study protocol was approved by the Medical Ethics Committee of the Fourth Hospital of Hebei Medical University.

1.2 Inclusion and Exclusion Criteria Diagnostic Criteria: Diagnosis followed the CSCO *Guidelines for the Diagnosis and Treatment of Esophageal Cancer* (2022 edition).

EC Group Inclusion Criteria: (1) Histopathologically confirmed ESCC; (2) Age 18–75 years; (3) No personal history of other malignant tumors; (4) No prior anti-tumor treatment (surgery, chemotherapy, radiotherapy, immunotherapy, etc.); (5) Signed informed consent.

Control Group Inclusion Criteria: (1) Age 18–75 years; (2) No malignant tumor history; (3) Signed informed consent.

Exclusion Criteria: (1) Infectious disease, antibiotic therapy, or probiotic use within two weeks; (2) Psychiatric disorders or lack of full civil capacity; (3) Severe cardiovascular disease or intestinal diseases (inflammatory bowel disease, irritable bowel syndrome, etc.).

1.3 Data and Sample Collection Clinical Data Collection: General information was collected from all participants, including gender, age, alcohol consumption history, smoking history, and clinical staging.

Sample Collection: Fresh fecal specimens were collected from both groups. Participants were instructed to defecate into clean containers, avoiding contamination with urine or toilet surfaces. Approximately 1g of fecal material from the middle to posterior portion of the sample was collected using a sterile spoon, placed in labeled cryovials, immediately flash-frozen in liquid nitrogen for approximately 1 minute, and then stored at -80°C. A total of 35 samples were collected from each group.

1.4 Gut Microbiota Detection and Analysis Genomic DNA was extracted from all 70 fecal samples. The V3–V4 region of the 16S rRNA gene was amplified using specific primers (forward: CCTAYGGGRBGCASCAG; reverse: GGACTACNNGGGTATCTAAT). PCR products were purified with magnetic beads, pooled in equal amounts based on concentration, and examined using 2% agarose gel electrophoresis. Target bands were recovered using a gel extraction kit, and libraries were constructed using a library preparation kit. Qualified libraries were sequenced on the NovaSeq6000 platform. Sequencing reads were assembled and subjected to quality control and filtering. Sequences were clustered into operational taxonomic units (OTUs) at 97% similarity, and OTU sequences were annotated against the Silva138 database. Based on the annotation results, alpha diversity, beta diversity, and inter-group comparisons were performed to reveal differences in community structure.

1.5 Statistical Analysis SPSS 22.0 software was used for statistical analysis. Normally distributed continuous data were expressed as mean ± standard deviation and analyzed using t-test if variance was homogeneous, or rank-sum test if variance was heterogeneous. Categorical data were analyzed using chi-square test. Statistical significance was set at $P<0.05$.

Results

2.1 Baseline Characteristics The EC group comprised 21 males and 14 females, while the control group included 18 males and 17 females. Age distribution in the EC group was: <50 years (4 cases), 50–60 years (11 cases), and >60 years (20 cases); in the DZ group: <50 years (7 cases), 50–60 years (15

cases), and >60 years (13 cases). Smoking history in the EC group included: never (10 cases), occasional (14 cases), and regular (11 cases); in the DZ group: never (15 cases), occasional (8 cases), and regular (12 cases). Alcohol consumption history in the EC group included: never (9 cases), occasional (17 cases), and regular (9 cases); in the DZ group: never (15 cases), occasional (13 cases), and regular (7 cases). Clinical staging in the EC group was: Stage I (3 cases), Stage II (6 cases), Stage III (17 cases), and Stage IV (9 cases). No significant differences were observed between the two groups in gender, age, smoking history, or alcohol consumption (P>0.05).

2.2 OTU Analysis of Gut Microbiota A total of 3,602 OTUs were obtained from the valid sequencing data. Species annotation identified 52 OTUs at the phylum level and 756 OTUs at the genus level. Based on these annotations, we compared the gut microbiota structure between ESCC patients and healthy controls. The top 10 most abundant species at phylum and genus levels were selected to generate stacked bar charts of relative abundance. At the phylum level, both groups were dominated by *Firmicutes*, *Actinobacteriota*, *Proteobacteria*, *Bacteroidota*, and *Verrucomicrobiota*. Notable differences were observed, with the EC group showing increased relative abundance of *Bacteroidota* and *Actinobacteriota* and decreased abundance of *Firmicutes* and *Verrucomicrobiota* compared to the DZ group [Figure 1: see original paper]A. At the genus level, the predominant taxa included *Bifidobacterium*, *Escherichia-Shigella*, *Faecalibacterium*, *Bacteroides*, *Prevotella*, *Klebsiella*, *Streptococcus*, *Blautia*, *Akkermansia*, and *Dialister*. Notably, *Faecalibacterium* abundance was significantly lower in the EC group compared to controls [Figure 1: see original paper]B.

2.3.1 Species Accumulation Curve Analysis Species accumulation boxplots describe whether species diversity increases with sample size, reflecting the rate of new OTU emergence during continued sampling. This analysis is primarily used to determine whether the sample size is adequate. The curves plateaued, indicating that the sample size was sufficient and the sequencing depth was appropriate, with no significant increase in microbial species expected with additional sampling [Figure 2: see original paper].

2.3.2 Alpha Diversity Analysis Alpha diversity reflects species richness and diversity. The Shannon and Simpson indices measure community diversity, while Chao1 and ACE indices estimate species richness. The Goods_{coverage} index assesses sequencing depth. These indices were used to analyze species distribution and richness between groups. No statistically significant differences were observed in any alpha diversity indices between the two groups. The Goods_{coverage} index exceeded 99% in both groups, confirming adequate sequencing depth (all P>0.05) [Figure 3: see original paper].

2.4 Beta Diversity Analysis Beta diversity compares microbial community composition between groups. Principal coordinate analysis (PCoA), based on

unweighted UniFrac distances, is a common method for beta diversity assessment—greater distances between samples indicate larger differences in community structure. PCoA plots revealed clear separation between EC and DZ groups, indicating substantial differences in microbial composition. T-test analysis of beta diversity further confirmed significant inter-group differences ($P<0.01$) [Figure 4: see original paper].

2.5.1 Inter-group and Intra-group Variation Analysis ANOSIM nonparametric test was used to determine whether inter-group differences exceeded intra-group variation, thereby validating the experimental grouping. Results demonstrated that inter-group differences were significantly greater than intra-group variation ($R=0.158$, $P<0.01$) [Figure 5: see original paper], confirming the validity of our groupings.

2.5.2 Genus-Level Differential Species Analysis At the genus level, 16 taxa showed significantly different abundance between groups. Among the top eight most abundant differential taxa, the EC group exhibited lower abundance of *Faecalibacterium*, *Roseburia*, and *Citrobacter*, while *Lactobacillus*, *Romboutsia*, *Ruminococcus_{torques}_{group}, Intestinibacter, and Turicibacter* were significantly enriched compared to the DZ group (all $P<0.05$) [Figure 6: see original paper]. Although *Lactobacillus* abundance was higher in the EC group, this was considered a confounding factor as more EC patients reported regular probiotic consumption (11 vs. 6 cases in the control group). Therefore, *Lactobacillus* was excluded from the differential analysis.

2.5.3 LEfSe Analysis LEfSe analysis identifies species with significantly different abundance between groups and evaluates the magnitude of their contribution to group differentiation. Histogram and cladogram results revealed 14 differentially abundant taxa. Specifically, the EC group showed elevated abundance of *Lactobacillales* (order), *Bacilli* (class), *Peptostreptococcaceae* (family), *Peptostreptococcales-Tissierellales* (order), *Romboutsia* (genus), and *Romboutsia ilealis* (species). Conversely, the DZ group exhibited higher abundance of *Veillonellaceae* (family), *Negativicutes* (class), *Veillonellales-Selenomonadales* (order), *Firmicutes* (phylum), *Faecalibacterium prausnitzii* (species), *Oscillospirales* (order), *Ruminococcaceae* (family), and *Faecalibacterium* (genus). At genus and species levels, *Faecalibacterium* and *F. prausnitzii* were significantly reduced, while *Romboutsia* and *R. ilealis* were markedly elevated in the EC group, representing important discriminative biomarkers ($P<0.05$) [Figure 7: see original paper].

Discussion

The human gut harbors approximately 100 trillion microorganisms weighing 1–2 kg, forming the largest symbiotic community in the body. These commensal microbes play crucial roles in nutrient absorption, immune regulation, maintenance

of intestinal barrier function, and gut homeostasis. In healthy individuals, *Firmicutes* and *Bacteroidetes* constitute approximately 90% of gut bacteria at the phylum level, with *Actinobacteria*, *Proteobacteria*, and *Fusobacteria* accounting for the remaining 10%. Numerous studies have demonstrated that gut microbiota influences tumorigenesis and progression through multiple mechanisms in various cancers.

In this study, no significant differences in alpha diversity (species richness) were observed between ESCC patients and healthy controls, whereas beta diversity (community structure) showed marked differences. Both groups were dominated by *Firmicutes*, *Actinobacteriota*, *Proteobacteria*, *Bacteroidota*, and *Verrucomicrobiota* at the phylum level. Compared to controls, ESCC patients exhibited increased abundance of *Bacteroidota* and *Actinobacteriota* and decreased abundance of *Firmicutes* and *Verrucomicrobiota*. T-test analysis identified 16 differentially abundant genera, with *Faecalibacterium*, *Roseburia*, and *Citrobacter* showing reduced abundance, while *Romboutsia*, *Ruminococcus_{torques}_{group}, Intestinibacter, *and* Turicibacter* were enriched in ESCC patients. LEfSe analysis revealed 14 differential taxa, with *Faecalibacterium* and *F. prausnitzii* significantly decreased, and *Romboutsia* and *R. ilealis* significantly increased in ESCC patients at genus and species levels.

Faecalibacterium was the most abundant and significantly altered genus in our study, potentially representing a key differential taxon in esophageal carcinogenesis. Previous research has reported reduced *Faecalibacterium* abundance in breast cancer patients, and its combination with phosphocholine metabolites may serve as a novel detection method for breast cancer. A Science study also found that *Faecalibacterium* was less abundant in melanoma patients unresponsive to PD-L1 therapy, suggesting it may enhance anti-PD-1 efficacy by upregulating CD8+ T cells and antigen presentation molecules. *F. prausnitzii*, the most prominent species in this genus, constitutes 5–15% of the normal human gut microbiota and is a major butyrate producer. Studies have shown significantly reduced *F. prausnitzii* in colorectal cancer patients, possibly related to decreased butyrate production. In vitro studies have demonstrated that *F. prausnitzii* and its supernatant inhibit colorectal cancer cell proliferation and promote apoptosis. Additionally, *F. prausnitzii* can suppress breast cancer progression by inhibiting IL-6 secretion and JAK2/STAT3 phosphorylation. These findings align with our results regarding *Faecalibacterium* and *F. prausnitzii*.

Studies by Zhang et al. and Seol et al. have reported elevated *Romboutsia* abundance in colorectal and gastric cancer patients, consistent with our findings. *Romboutsia ilealis* has been identified as a potential aggravating factor in glucose metabolism and a risk factor for type 2 diabetes, though its relationship with cancer remains largely unexplored. Wang et al. found that *Roseburia* was significantly reduced in colorectal cancer patients, possibly through butyrate-mediated inhibition of NF- B pathway activation. Zheng et al. reported increased *Ruminococcus* abundance in lung cancer patients. Tumor necrosis factor

can directly kill and inhibit tumor cells, and Lida et al. found that *Ruminococcus* positively correlated with TNF-α secretion, collectively indicating that gut microbiota alterations are closely related to cancer development, progression, and therapy. Notably, the trend of *Ruminococcus* change is consistent with our previous findings in an orthotopic esophageal cancer mouse model, providing mutual validation. However, some differential taxa differed from previous reports, likely because human gut microbiota is influenced by numerous external factors including diet, disease, and medication. For instance, although *Lactobacillus* is considered a probiotic and was more abundant in our ESCC patients, this contradicted previous studies. Upon inquiry, 11 ESCC patients reported regular consumption of probiotic dairy products compared to only 6 controls, despite no probiotic use within two weeks prior to sampling. This suggests that future studies should extend the probiotic washout period.

This study has several limitations. First, all patients were from a single hospital, representing a geographically homogeneous population with a relatively small sample size; multi-center, large-scale studies are needed for validation. Second, 16S rDNA sequencing primarily investigates community diversity, species composition, and phylogenetic relationships but cannot provide in-depth analysis at the gene and functional levels like metagenomic sequencing. Third, our study only revealed associations between ESCC and gut microbiota alterations without mechanistic validation. Finally, there is currently no standardized washout period for antibiotics and probiotics prior to sampling; based on our findings, this period should be extended, though the optimal duration requires further investigation. These limitations will guide our future research directions.

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Note: Figure translations are in progress. See original paper for figures.