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Associations of oral and intestinal florae and serum inflammatory factors with pathogenesis of oral cancer

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Abstract. – OBJECTIVE: The aim of this study was to explore the effects of oral and intestinal florae and serum inflammatory factors on the pathogenesis of oral cancer.

PATIENTS AND METHODS: Oral cancer patients and healthy subjects in our hospital were enrolled in disease group (n=50) and control group (n=50), respectively. Oral flora of subjects was collected using the sterile cotton swab. Microbial deoxyribonucleic acid (DNA) was extracted for Polymerase Chain Reaction (PCR) amplification and sequencing. Subsequently, the feces were also collected from patients, and sent to the company for analysis of microbial composition via sequencing. In addition, the levels of serum inflammatory factors tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8), IL-6, and IL-1β in disease group and control group were detected via enzyme-linked immunosorbent assay (ELISA).

RESULTS: The number of patients with a history of drinking (*p*=0.040) and betel nut chewing (*p*=0.000) in the disease group was larger than that in the control group, and the difference was statistically significant. In terms of oral flora distribution, the ratios of dominant bacteria *Staphylococcus* and *Rothia* were 64% and 50% in disease group, which were significantly higher than those in the control group (24% and 18%) (*p*=0.023 and 0.034). In terms of intestinal flora distribution, the abundance of intestinal florae (*Flavobacteriaceae, Sphingobacteriales, Rikenella, Pseudomonadales, Tetragenococcus and Acinetobacter*) in the disease group was remarkably higher than that in the control group (*p*<0.05). However, the abundance of *Vagococcus* and *Pediococcus* in control group was significantly higher than that in the disease group (*p*<0.05). Among intestinal flora, *Firmicutes* exhibited a highly positive correlation with *Bacteroides* (r=0.341, *p*=0.023), and a highly negative correlation with *Ruminococcus* (r=-0.832, *p*=0.000). *Bacteroides* had a highly negative correlation with Lactobacillus (r=-0.763, *p*=0.000) and *Enterococcus* (r=-0.461, *p*=0.000). In disease group, the levels of TNF-α (*p*=0.021), IL-8 (*p*=0.000), and IL-1β (*p*=0.000) were evidently higher than those in the control group [(23.51±2.14) ng/L *vs.* (12.34±2.45) ng/L, (89.75±4.29) ng/L *vs.* (43.23±3.25) ng/L, (42.25±3.25) ng/L *vs.* (15.32±1.47) ng/L]. However, there was no statistically significant difference in IL-6 level between the two groups (*p*=0.217).

CONCLUSIONS: Oral and intestinal florae and serum inflammatory factors are associated with the pathogenesis of oral cancer.

Key Words:
Oral flora, Intestinal flora, Oral cancer.

Introduction

Oral cancer is a malignant tumor in the oral cavity, which occurs in lips, tongue, mucous membrane or other non-specific sites[1,2]. As one of the top ten most common cancers, oral cancer frequently occurs in Southeast Asia or Central and Eastern Europe[3]. In China, the incidence rate of oral cancer is about 6-8/100,000. Meanwhile, the number of male patients is about three times than that of female patients[4]. The occurrence of oral cancer is mainly related to the changes in oral microenvironment caused by various factors, leading to abnormal changes in cells and the activation of oncogenes[5]. Chewing betel nut is one of the most common external factors for oral cancer[6]. Therefore, it is of great significance to find the association between the changes of oral microenvironment and the pathogenesis of oral cancer.

Microorganisms play an important role in the normal physiological and pathological processes in the human body. They have been confirmed to be correlated with the pathogenesis of many dis-

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eases, such as asthma[7], type 2 diabetes mellitus[8], and colitis[9]. Oral and intestinal microorganisms can affect local oral and intestinal microenvironment. Meanwhile, they are also affected by external factors, including alcohol and nicotine, which may be intermediate factors for the occurrence of oral and intestinal diseases[10-12]. Besides, the occurrence of oral cancer may also be related to the changes in the composition of oral or intestinal flora.

In this paper, therefore, the number of people with a history of smoking, drinking, and betel nut chewing was compared between 50 oral cancer patients and 50 healthy people. Combined with the abundance of oral and intestinal florae, the changes in the serum levels of inflammatory factors were detected. Meanwhile, the influence of oral and intestinal florae and serum inflammatory factors on the pathogenesis of oral cancer was explored. Our findings might help to clarify the possible pathogenesis of oral cancer.

Patients and Methods

General Data

A total of 50 oral cancer patients treated in our hospital in the last year (disease group) were enrolled as research subjects. Meanwhile, 50 healthy people in the health management center (control group) were collected as normal controls. In the disease group, there were 32 males and 18 females with an average age of (48.43±4.35) years old. In the control group, there were 30 males and 20 females with an average age of (51.35±3.84) years old. There were no statistically significant differences in age and sex distribution between the two groups ($p>0.05$). Clinical data, such as the history of smoking, drinking and betel nut chewing, past medical history and history of drug allergy, were collected in both groups. In the disease group, all patients were diagnosed with oral cancer *via* observation of cancer tissue sections by senior pathologists. This investigation was approved by the Ethics Committee of Liaocheng People's Hospital.

Detection of Oral Flora

Oral flora was collected from all subjects at 4 h after the meal. Before collection, the subjects in both groups gargled with clean water for 3 min to remove food residues. After the oral cavity was slightly dry, oral cancer tissues or normal oral mucosa was sampled using the sterile cotton swab. Subsequently, collected tissues were placed into the special detection solution for flora. Then, the samples were sent to Nanjing Biotechnology Co., Ltd. (Nanjing, China), and microbial deoxyribonucleic acid (DNA) was extracted for Polymerase Chain Reaction (PCR) amplification and sequencing. Finally, the composition and abundance of oral flora were detected and analyzed in both groups.

Detection of Intestinal Flora

Feces were collected in both groups and cryopreserved in a liquid nitrogen container, followed by detection of intestinal flora. Collected samples were sent to Nanjing Biotechnology Co., Ltd. for analysis of intestinal microorganisms. After extraction, amplification, establishment of database and labeling of microbial genomic DNA, high-throughput sequencing was performed using Illumina MiSeq and Ion PGM. The species and relative abundance of microorganisms in the feces were detected. The species of bacteria were determined, and the detection rate of bacteria was calculated. Bioinformatics analysis was conducted for intestinal flora in both groups in the Galaxy website (http://huttenhower.sph.harvard.edu/galaxy/). The data of intestinal flora were uploaded to the website for LEfSe and linear discriminant analysis. Finally, the composition of intestinal flora in both groups was obtained, and the data were visualized.

Detection of Levels of Serum Inflammatory Factors

The changes in the serum levels of inflammatory factors tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8), IL-6 and IL-1β in both groups were detected according to the instructions of enzyme-linked immunosorbent assay (ELISA) kits (R&D, Minneapolis, MN, USA). Peripheral blood was drawn from the subjects and centrifuged at 3,500 rpm for 5 min. Subsequently, the upper-layer serum was collected for detection. Absorbance at 450 nm was measured using a micro-plate reader (Bio-Rad, Hercules, CA, USA), with 3 replicates in each group. Next, they were converted into the actual concentrations of TNF-α, IL-8, IL-6 and IL-1β through standard curves. The average sensitivity of the test was <0.47 pg/mL, and the inter-batch coefficient of variation was 6.4%.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 23.0 software (IBM Corp., Armonk, NY, USA) was used for statistical processing. Chi-

Table I. Comparison of number of people with a history of smoking, drinking and betel nut chewing between the two groups.

Group	n	Smoking	No smoking	Drinking	No drinking	Betel nut chewing	No betel nut chewing
Control group	50	19	31	14	36	4	46
Disease group	50	28	22	30	20	40	10
χ^2		5.35	6.43	16.45			
p		0.069	0.040	0.000			

square test and *t*-test were used for the comparison of enumeration data and measurement data, respectively. Pearson method was selected for correlation analysis. $p<0.05$ was considered statistically significant.

Results

Comparison of Number of People With a History of Smoking, Drinking and Betel Nut Chewing Between the Two Groups

As shown in Table I, the number of people with a history of drinking ($p=0.040$) and betel nut chewing ($p=0.000$) in disease group was larger than that in control group, and the difference was statistically significant.

Analysis of Oral Flora in Both Groups

As shown in Table II, in terms of oral flora distribution, the ratios of dominant bacteria *Staphylococcus* and *Rothia* were 64% and 50% in disease group, which were significantly higher than those in the control group (24% and 18%) ($p=0.023$ and 0.034).

Analysis of Intestinal Flora in Both Groups

In terms of intestinal flora distribution, the abundance of intestinal florae (*Flavobacteriaceae*, *Sphingobacteriales*, *Rikenella*, *Pseudomonadales*, *Tetragenococcus* and *Acinetobacter*) in disease group was significantly higher than that in the control group ($p<0.05$). However, the abundance of *Vagococcus* and *Pediococcus* in the control group was remarkably higher than that in disease group ($p<0.05$) (Figures 1 and 2).

Correlation Analysis of Intestinal Flora

Among intestinal florae, *Firmicutes* exhibited a highly positive correlation with *Bacteroides* ($r=0.341$, $p=0.023$), and a highly negative correlation with *Ruminococcus* ($r=-0.832$, $p=0.000$).

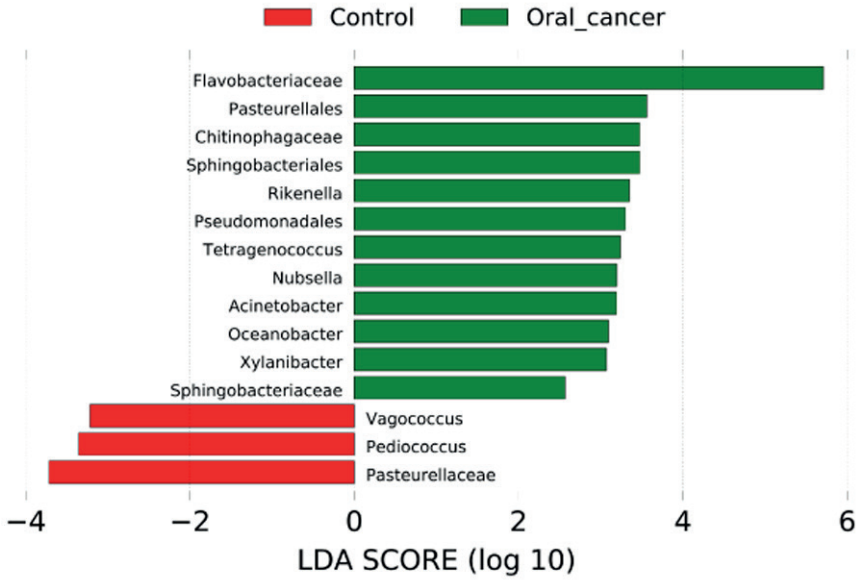

Figure 1. LDA score of intestinal flora in both groups

Table II. Analysis of oral flora in both groups [n (%)].

	n	Streptococcus	Staphylococcus	Lactobacillus	Rothia	Others
Control group	50	21 (42)	12 (24)	15 (30)	9 (18)	4 (8)
Disease group	50	16 (32)	32 (64)	12 (24)	25 (50)	6 (12)
χ^2		5.32	9.42	12.35	8.65	3.32
p		0.150	0.023	0.503	0.034	0.345

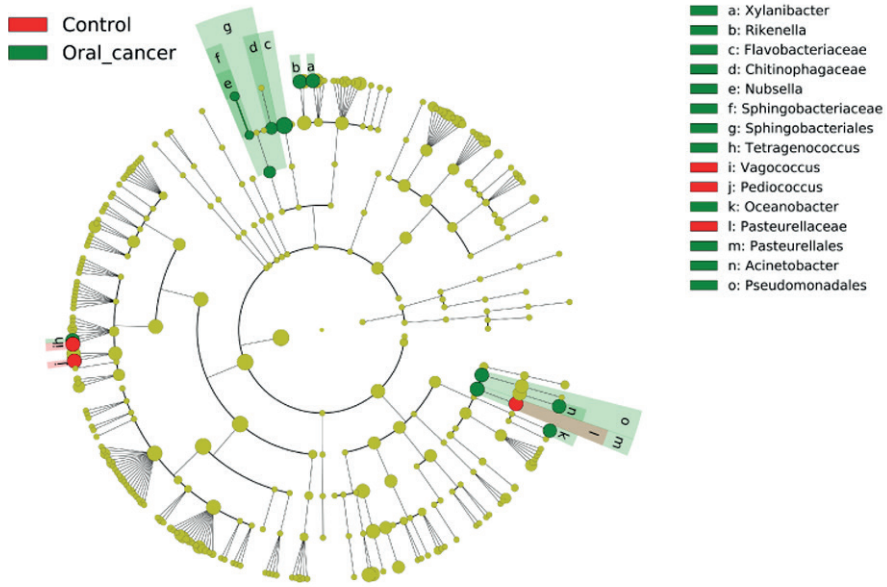

Figure 2. LEfSe analysis of intestinal flora in both groups.

However, *Bacteroides* had a highly negative correlation with *Lactobacillus* (r=-0.763, p=0.000) and *Enterococcus* (r=-0.461, p=0.000) (Figure 3).

Comparison of Levels of Serum Inflammatory Factors between the Two Groups

In disease group, the levels of TNF-α (p=0.021), IL-8 (p=0.000), and IL-1β (p=0.000) were evidently higher than those in the control group [(23.51±2.14) ng/L *vs.* (12.34±2.45) ng/L, (89.75±4.29) ng/L *vs.* (43.23±3.25) ng/L, (42.25±3.25) ng/L *vs.* (15.32±1.47) ng/L]. However, there was no statistically significant difference in IL-6 level between the two groups (p=0.217) (Table III).

Discussion

As a common cancer closely related to the changes in oral microenvironment, the morbidity and mortality rates of oral cancer increase with age. This may be related to dietary habits, such as smoking, drinking, and betel nut chewing[13,14]. Arecoline, nicotine, and alcohol in food can stimulate oral mucosa and lead to long-term inflammation. Chronic inflammation is considered as

Table III. Comparison of levels of serum inflammatory factors between the two groups.

	n	TNF-α (ng/L)	IL-8 (ng/L)	IL-6 (ng/L)	IL-1β (ng/L)
Control group	50	12.34±2.45	43.23±3.25	56.43±4.85	15.32±1.47
Disease group	50	23.51±2.14	89.75±4.29	50.21±5.94	42.25±3.25
p		0.021	0.000	0.217	0.000

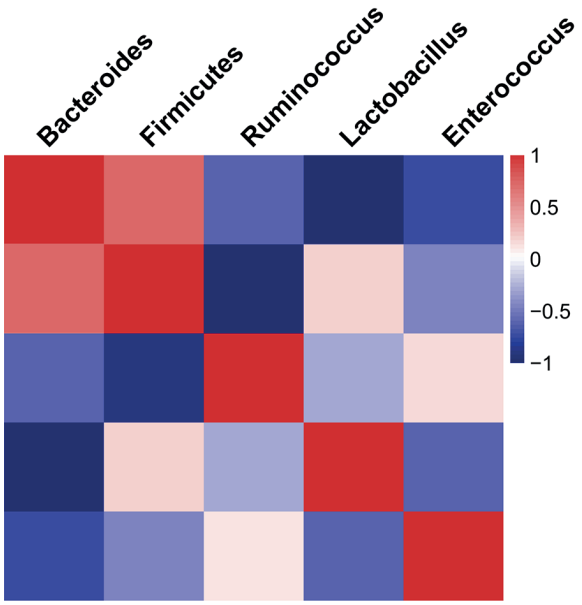

Figure 3. Pearson correlation analysis of intestinal flora.

an important factor for oral cell canceration[15,16]. It has also been proved that smoking and drinking are co-promoters for oral cancer[17]. In addition, the pathogenesis of oral cancer is related to the lack of nutrients in the body. Fathi et al[18] have demonstrated that the deficiencies of vitamin and iron are important factors promoting the occurrence of oral cancer. Long-term stimulation of various harmful substances against the oral cavity or the lack of nutrients may cause changes in oral microenvironment, serving as an important cause of oral cancer. Therefore, exploring the changes in local oral microenvironment and the systemic changes in the body during the onset of oral cancer is important for revealing its pathogenesis. In this study, the number of people with a history of smoking, drinking, and betel nut chewing was counted in disease group and control group, respectively. It was found that the number of people with a history of drinking (p=0.040) and betel nut chewing (p=0.000) in disease group was significantly larger than that in control group, and the difference was statistically significant. The above findings proved once again that drinking and betel nut chewing promoted the occurrence of oral cancer. However, their specific influence on the oral microenvironment remained to be further studied.

Oral flora refers to normal microorganisms colonized in the oral cavity, which exerts an important regulatory effect on material decomposition, local oral microenvironment homeostasis and immune balance[19]. The oral cavity is an absolutely bacterial environment, in which microorganisms include both beneficial and harmful bacteria. The disorders of the proportion and abundance of different bacteria may be an important cause of the disease[20]. In this study, in terms of oral flora distribution, the ratios of dominant bacteria *Staphylococcus* and *Rothia* were 64% and 50% in the disease group, which were both significantly higher than those in control group (24% and 18%) (p=0.023 and 0.034). It can be seen that *Staphylococcus* and *Rothia* in the oral cavity of patients may play important roles in the occurrence and development of oral cancer. Meanwhile, they may affect cancer cell proliferation or differentiation *via* affecting local oral microenvironment.

Intestinal flora refers to important microorganisms in the digestive tract. The changes in its composition and abundance affect the local physiological and pathological processes of the intestine. This may be related to the disorders of metabolite decomposition and the imbalance of intestinal microenvironment[21]. It is generally believed that intestinal microorganisms are mostly related to the occurrence of local intestinal diseases, such as ulcerative colitis and colon cancer. However, they were not highly correlated with extra-intestinal diseases[22]. In this study, in terms of intestinal flora distribution, the abundance of some intestinal florae (*Flavobacteriaceae*, *Sphingobacteriales*, *Rikenella*, *Pseudomonadales*, *Tetragenococcus* and *Acinetobacter*) in the disease group was significantly higher than that in the control group (p<0.05). However, the abundance of *Vagococcus* and *Pediococcus* in the control group was remarkably higher than that in disease group (p<0.05). Among intestinal flora, *Firmicutes* had a highly positive correlation with *Bacteroides* (r=0.341, p=0.023), and a highly negative correlation with *Ruminococcus* (r=-0.832, p=0.000). *Bacteroides* had a highly negative correlation with *Lactobacillus* (r=-0.763, p=0.000) and *Enterococcus* (r=-0.461, p=0.000). The above results demonstrated that intestinal flora might be of great significance in the pathogenesis of oral cancer. The changes in intestinal flora may be one of the important causes of oral cancer, but its specific mechanism needs further research.

Finally, the levels of serum inflammatory factors were compared between the disease group and control group. The results showed that in the disease group, the levels of TNF-α (p=0.021), IL-8 (p=0.000) and IL-1β (p=0.000) were evidently higher than those in the control group [(23.51±2.14) ng/L *vs.* (12.34±2.45)

ng/L, (89.75±4.29) ng/L *vs.* (43.23±3.25) ng/L, (42.25±3.25) ng/L *vs.* (15.32±1.47) ng/L]. However, there was no statistically significant difference in IL-6 level between the two groups (p=0.217).

Conclusions

The novelty of this investigation was that the occurrence of oral cancer may be related to the levels of inflammatory factors in the body, and our findings could help to illuminate the possible pathogenesis of oral cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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