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Risk on Dental Porphyromonas with Esophageal Cancer: A Guangdong Elderly Cohort

Study

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Abstract:

Objective: To investigate the relationship between Dental Porphyromonas Concentration and Esophageal Cancer in Guangdong, China.

Methods: A total of 2031 research objects were included from 2016 and follow-up for 6 years, greater than 65 years old, all are from outpatient department with esophageal foreign body sense. Data are retrospectively analyzing in year 2021. There were 43 cases found to developed esophageal cancer, 7 of them are from exposure group, (high dental porphyromonas group) and 36 from control. Two groups of individuals were retrieved from the Guangdong Provincial Hospital database between year 2016 June to 2021 June. Age, gender, race and region are strictly matched. Both of case and the controls are processing through: a. Specimen collection, b. DNA extraction, c. High-throughput sequencing (screening stage), d. Quantitative determination of target bacteria in the samples of the experimental group by Q-PCR, e. Statistical analysis of experimental data. Two sample t-test are using to detect whether have difference on concentration of Dental Porphyromonas between cases and control group. SAS 15 windows version is used as analytic tools.

Results: We found that the odds on having Esophageal Cancer from high dental porphyromonas group is 1.23 times greater than controls (OR=1.23). Also, mean of concentration on Dental Porphyromonas in Esophageal Cancer Group (Cases) are statistically higher than Healthy controls, with a mean of 0.0157 in cases versus 0.0025 in controls, which is 6.54 times higher in Esophageal Group, compare with control group, with P<0.001. Where Hot Tea Drinking Habit are found associated with

Esophageal Cancer, with the Odds Ratio (OR) on 1.30, as well as current smoking on an 1.13 odds ratio.

Conclusion: The cohort study had found some significant associations on high Dental Porphyromonas with Esophageal Cancer Patient, where this association still lacks of proof to be a determinate causal relationship. Further greater populational longitudinal studies are need to carry out to find out more causal association, as well as finding out Dental Porphyromonas relevant Social Determinant on Esophageal Cancer.

Keywords: Dental Porphyromonas Concentration, Esophageal Cancer, High-throughput sequencing, Cohort, Odds Ratio

Introduction:

China is a country with a high incidence of esophageal cancer in the world, among which TaiHang mountain area in Henan province and Chaoshan area in Guangdong province are found to have the highest prevalence. The prognosis of early esophageal cancer after radical resection was fair-good, and its 10-year survival rate was up to 95%. However, as a matter of fact, most patients with esophageal cancer are in the middle and advanced stage when they see a doctor, a considerable number of patients have lost the opportunity of surgical process, and the 5-year survival rate is lower than 10% [1]. Therefore, it is very important to improve the early diagnostic rate of esophageal cancer, in order to improve the prevention on esophageal cancer.

At present, the exact cause of esophageal cancer is not clear. In recent years, microecology, as a theory explaining the role of bacteria in tumor pathogenesis, has become a hot topic. In 2013, Aleksandar Kostic and Mara Roxana Rubinstein's research team used intestinal tumor to form mouse model, also with human colon cancer cells, confirmed that clostridia nucleosa induced pro-inflammatory reaction and carcinogenic activity, which promoted the growth of colorectal cancer [2, 3]. In 2012, James J Farrell compared the saliva microflora of pancreatic cancer patients and healthy people, found out that salivary microflora showed significant changes in pancreatic cancer, indicating that salivary bacteriological changes have predictive

diagnostic value in pancreatic cancer [4]. In 2009, Yang et al. found that there have difference in microflora of esophagus between different disease states, including normal esophagitis dominated by streptococcus, esophagitis and Barrett(BE) esophagus, which dominated by gram-negative anaerobe [5]. Histopathological changes of esophageal cancer, especially squamous cell carcinoma, have not been reported. Since most of the esophageal bacteria come from the mouth, it is speculated that oral bacteria are correlated with esophageal cancer. There have no large reports been announced regarding the changes and clinical significance on bacteria in saliva with Esophageal Cancer.

Esophageal squamous carcinoma patient is as the research object of this study, patient's saliva and organization are collected and use DNA sequencing to study correlation with tumor and microbial flora, which compared with the normal controls. Prediction model is set up, to explore a simple, noninvasive, and cheap Esophageal Cancer diagnostic tool, in order to achieved the goal of early detection and early prevention of esophageal cancer.

(ii) current situation at home and abroad

1. Bacteria and tumor

Bacteria are closely related to gastrointestinal tumors. Recently, Johan Dicksv reported that patients with gastric cancer had a large number of streptococcus, lactobacillus, veronella, prevotella, bleeding bacillus and neisseria in the stomach, which was different from normal people and chronic gastritis [6]. It is known that rhodococcus aeruginosa and bacillus sanguineus are rich in nitrite reductase and nitrite reductase, which can produce n-nitrosamines, n-nitrosamines and other carcinogens. Jiyoung Ahn et al. also found that the diversity of intestinal flora and the species of clostridium difficile decreased in patients with colon cancer. Clostridium difficile can degrade dietary fiber into butyrate, which can prevent colon cancer and digestive tract inflammation. Meanwhile, clostridium and porphyromonas are increased, which are related to the increase of oral and digestive tract inflammation. Aleksandar Kostic and Mara Roxana Rubinstein et al. also found in in-depth studies that clostridium nucleate can induce the bacteria-specific cell expression elements of inflammation and tumorigenesis, which are called FadA elements. Combining with the e-cadherin molecule of colorectal cancer cells, they can activate the in-cell -catenin signal and regulate the differential inflammatory and carcinogenic responses [2, 3].

The function of bacteria in saliva contains complex molecular substances similar to blood, including a variety of enzymes, hormones, antibodies, anti-microbial components and growth factors, etc. [7]. These molecular substances are transported into saliva via transmembrane transport within salivary gland cells or gap junction between cells in peripheral blood circulation [8]. Therefore, saliva is called "a mirror of human health", which can reflect various diseases of the body, such as cancer [4, 9, 10], infectious diseases [11], cardiovascular diseases [12] and cerebrovascular diseases [13].

In recent years, due to the progress of molecular biology research technology, such as the application of high-throughput gene chip and second-generation sequencing technology, it is found that there are as many as 700 kinds of bacteria in the human mouth, 35-50% of which cannot be cultured by traditional methods, and these types of bacteria that cannot be cultured may be related to the occurrence of human health and diseases. The vast majority of bacteria in the saliva of normal healthy adults belong to the genera of streptococcus, platycoccus, neisseria, clostridium, vironella and Aggregatibacter. There are close and complex interactions between these oral microbial communities and between microbial communities and the host, which maintain the health of the host. Therefore, the analysis of microbial diversity and specificity in saliva is of great significance for the diagnosis, prediction and prevention and treatment of diseases related to human body, just like the study of gastrointestinal bacteria [14, 15].

DL Mager detection of oral squamous cell carcinoma patients with saliva and found three kinds of high levels of bacteria, respectively is gum carbon dioxide eosinophilic fiber bacteria, black fungus and light weight throw shooter, predictive diagnostic sensitivity and specificity of oral cancer are 80% above, put forward the saliva microorganism forecast of oral squamous cell carcinomas had a very good application prospect [10]. HEBA. S.S aid and other studies have found that inflammatory state of inflammatory bowel disease (IBD) is associated with saliva microbial flora imbalance, many inflammatory factors and IgA increased in patients with saliva, and lysozyme is reduced, and these factors and lysozyme, IL - 1 beta streptococcus in saliva, platts bacteria genera, haemophilus species and WeiRong coccus is closely related to increased levels of [17], the result that a connection between IBD and saliva microorganisms.

In conclusion, so far studies have proved that diseases related to oral/saliva bacteria include oral cancer [10, 18], pancreatic cancer [4], inflammatory bowel disease [17],

celiac disease [19], and cardiovascular disease [20-22]. American society for microbiology research recently reported pancreatic cancer patients with oral cavity there are significant differences between microbial components and healthy controls, patients with pancreatic cancer in the group of oral microorganisms leptothrix and campylobacter is significantly higher than other groups, while the other three kinds of oral microorganism's streptococcus, treponema and WeiRong coccus is significantly lower than other groups. To sum up, salivary microflora, as a predictive biomarker of systemic diseases, especially digestive tract diseases, may have research value.

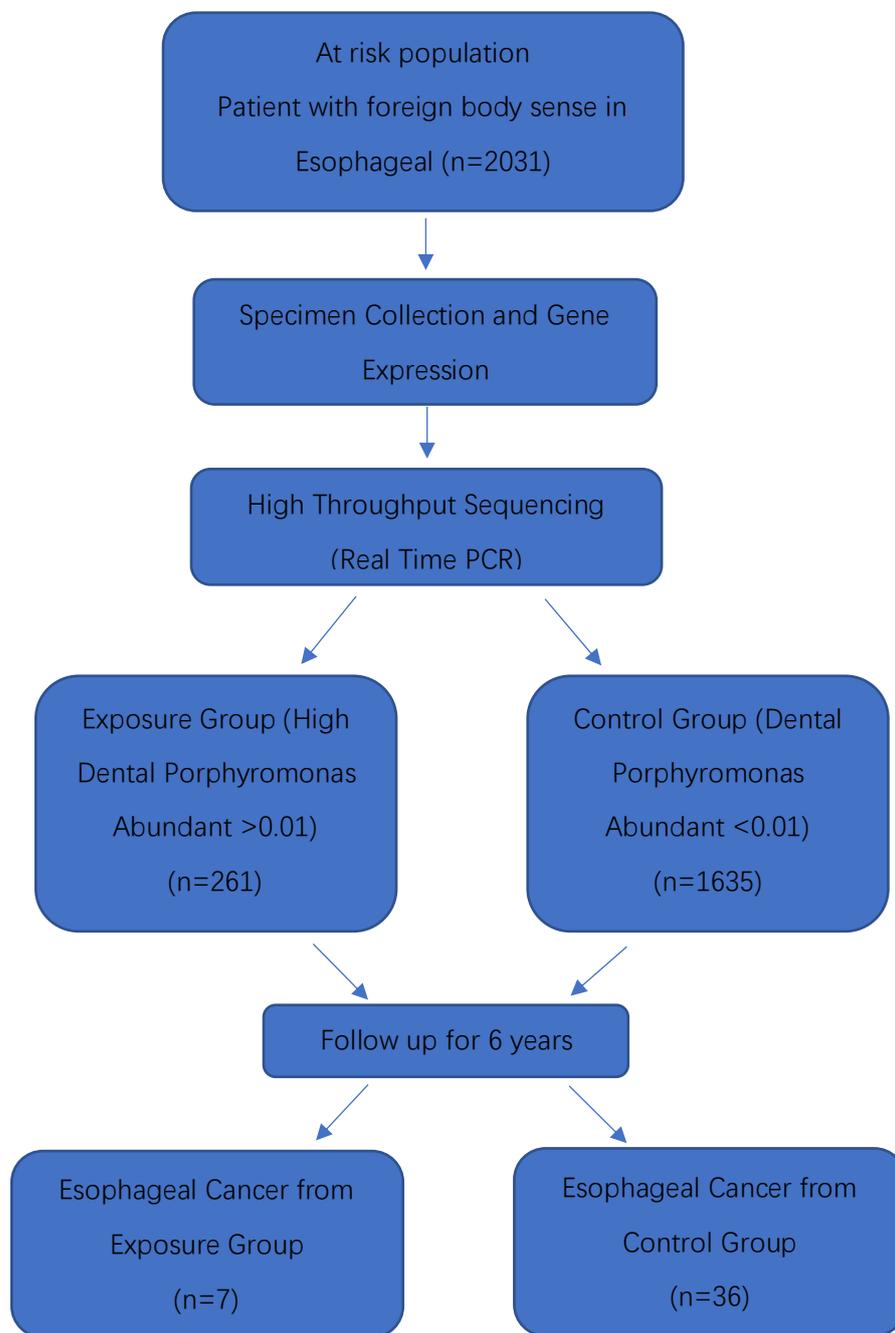
This research will collect saliva and esophageal squamous cancer and normal esophageal tissue and normal tissue, using 16 s rDNA / 16 s rRNA technology testing saliva and bacteria in the organization, compared the difference of esophageal cancer patients and normal salivary bacteria and the bacteria and the esophagus tissues bacteria in the saliva of different, find the difference of target bacteria, validation of this group of bacteria through the Q - PCR technology in the diagnosis of esophageal cancer in forecasting value, establish discriminant model, further in ShanTou city of high incidence of high-risk patients to detect the model predictive diagnostic value.

The collection of saliva is simple, convenient and non-invasive and will not cause anxiety or discomfort. Since patients with esophageal cancer have symptoms of esophageal obstruction and reflux, local bacteria in the esophageal tissue will enter the oral cavity with reflux to colonise and reproduce, and remain in the saliva for a long time, we speculated that salivary bacteriological detection should be of strong rationality and practicality in the prediction and diagnosis of esophageal cancer and related etiology studies.

The relationship between bacteriology (including saliva and bacteria in tissues) and esophageal squamous cell carcinoma has not been reported at home and abroad, and the study on the prevention and early diagnosis of esophageal carcinoma in shantou, guangdong province is of great value and social significance. Therefore, further study will have a broad clinical application prospect.

Methodology:

Conceptual Framework and Inclusion Flow chart of the study:



a. Research Method:

Research objects were at risk population, which is Patient greater than 65 years old, with foreign body sense in Esophageal (n=2031) and follow up for 6 years.

Those with High Dental Porphyromonas were defined as Exposures Group (DNA Abundant >0.01), while Normal Dental Porphyromonas (DNA Abundant >0.01) were defined as Control Group. Two groups of individuals were retrieved from the Guangdong Provincial Hospital database between year 2016 June to 2021 June. Age, gender, race and region are matched. Endoscopy and pathology are being performed for confirmation on Esophageal cancer patient.

b. Inclusion criteria:

Those with High Dental Porphyromonas were defined as Exposures Group (DNA Abundant >0.01), while Normal Dental Porphyromonas (DNA Abundant >0.01) were defined as Control Group.

Confirmation on Diagnosis: 1) endoscopy and pathology confirmed patients with esophageal cancer 2) No other organic or systemic (e.g., diabetes, hepatitis, etc.) and No oral disease.

c. Exclusion criteria:

1) Patient had a history of diarrhea or infectious diseases in the past one week; 2) drugs used to regulate intestinal flora and affect metabolism in the past four weeks; 3) antibiotics or PPI or other acid-inhibiting drugs used in the past eight weeks; 4) postoperative and chemoradiotherapy.

c. Sample Size

The sample size of this study were 2031 subjects total, 261 from exposure group (high Dental Porphyromonas), with 1635 controls (Normal Dental Porphyromonas). The sample size is considered to fulfill the expect effect size of 2 times better outcome of treatment, from previous meta-analysis with systematic review, under a two tails type one error ($\alpha < 0.05$) level. Calculating by Sample Size Calculator on SurveyMonkey.com, the Statistical Power is found to be 92% when using 600 as our study sample size.

d. Protection of Human Subjects

The Elderly subjects in the study would likely have concerns about the consequences of their participation and confidentiality of medical and personal information. Those eligible and selected for participation in the study would be informed that their participation is voluntary and that they are able to withdraw from the study at any time without repercussions. Chinese IRB approval (hospital level, Non experimental trial) was obtained before study begin.

e. Laboratory procedure:

Saliva DNA extraction: With UltraClean[®] Microbial DNA Isolation Kit (the United States, the MOBIO) extract bacterial DNA, DNA extraction method carried out in accordance with the Kit instructions.

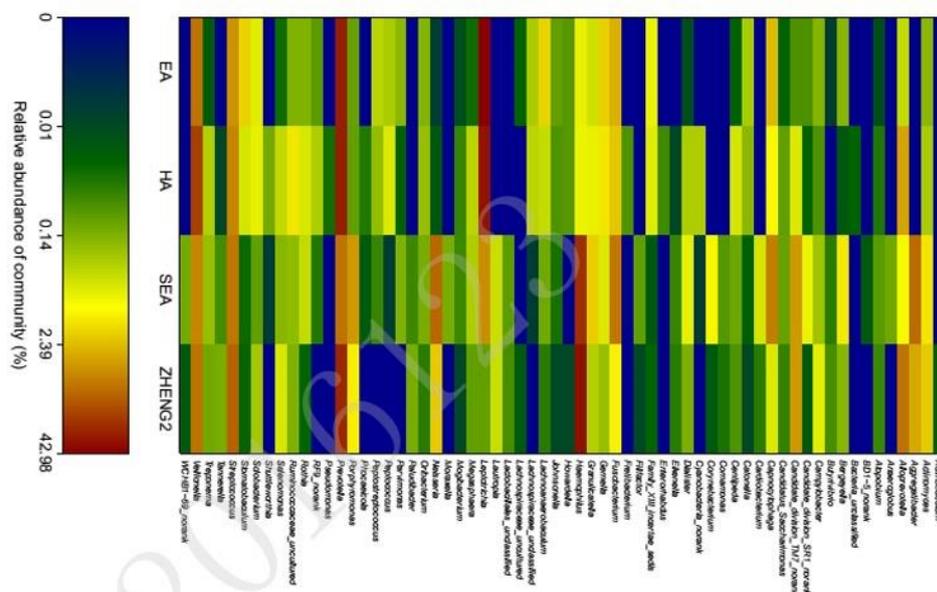
1. DNA quality detection: The extracted DNA should meet the following standards: DNA concentration 5ng/ul, total DNA 150ng; OD value (od260/280) was 1.8 ~ 2.0. According to the results of agarose gel electrophoresis, the DNA quality was evaluated. An appropriate amount of the sample was then placed in the centrifuge tube and diluted with sterile water to 1ng/ul.

- OTU clustering and analysis: All Effective Tags of all samples are clustered using Uparse software (Version 7.0.1001), and sequences are implicitly clustered into OTUs (Operational Taxonomic Units) with 97% Identity. Meanwhile, representative sequences of OTUs are selected according to its algorithm principles. What is screened is the sequence with the highest occurrence frequency in OTUs as the representative sequence of OTUs.
- Species annotation: The OTUs representative sequences were annotated for species, and RDP Classifier (Version 2.2) method and GreenGene database were used for species annotation analysis (threshold was set as 0.8~1), and the community composition of each sample was counted at each classification level: (kingdom), (phylum), (class), (order), (family), (genus), (species).

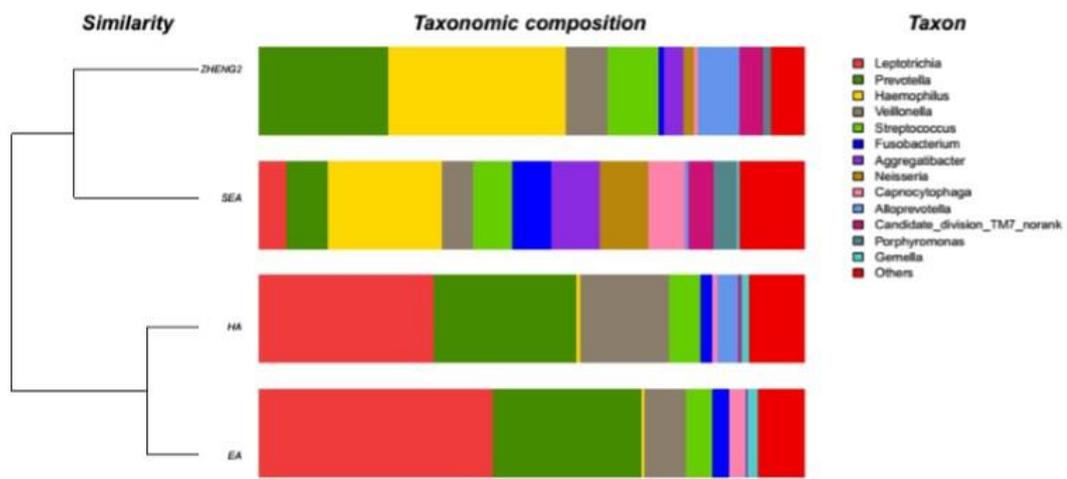
The study is funded by Guangdong medical science and technology research funding: A2016123.

China-IRB approval are obtained before the study regarding having relevant procedure on patients.

Heatmap:



TreeBar:



f. Statistical Analysis:

STATA 15 windows version are using as statistical analysis tool. Multi-variate Regression were performed for comparison on association regarding Dental Porphyromonas with onset with Esophageal Cancer, The mean comparison of Dental Porphyromonas Concentration between Esophageal Cancer group and controls is using two sample t-test with unequal variance, while the relationship between Hot Tea Drinking Habit with Esophageal Cancer is using chi-square test. The test on association between age with Dental Porphyromonas Concentration are using linear regression model.

Result:

From table 1 we can see demographics of the study. The mean age of Esophageal cancer group is 51.7 where controls are 48.5 years. There are 69.7% of male in Esophageal Group while 67.5% of which in Controls. 20.9% of esophageal cancer patients have oral infections and 16.3% of controls also having similar problem. 62.8% of disease groups are current smokers while the proportions are 67.5% in controls, respectively. 44.2% of disease patients are reported alcohol drinking, while 37.2% of controls reports also. There has a hot tea drinking habit in Guangdong and Fujian Provinces, we also include the hot tea drinking habit questionnaire. 32.5% of disease group are reported with hot tea drinking habit, while 27.9% of controls reported also, with a non-statistical P value result 0.532.

Table 1: Sociodemographic

	Exposure Group	Control Group	P
	(n=261)	(n=1635)	value
Age (mean years, SD)	66.7 (6.9)	68.5 (7.2)	0.79
Gender			0.28
Male	167 (64.1%)	1105 (67.6%)	
Female	94 (35.9%)	530 (32.4%)	
Oral Infection Status			0.21
Yes	58 (22.2%)	202 (18.3%)	
No	203 (77.8%)	1433 (81.7%)	
Current Smoking Status			0.488
Yes	171 (65.9%)	1103 (67.5%)	
No	90 (34.1%)	532 (32.5%)	
Daily Alcohol using			0.379
Yes	120 (45.9%)	641 (39.2%)	
No	141 (54.1%)	994 (60.8%)	
Hot Tea Drinking Habit			0.032
Yes	88 (33.7%)	458 (28.0%)	
No	173 (66.3%)	1177 (73.0%)	

We perform a linear regression model between age and concentration on Dental Porphyromonas. From Figure 1 we can see a roughly linear association between age and concentration of Dental Porphyromonas. Then we use Linear regression model to test with the association. Result showing that the coefficient of linear regression is 513.86, the linear association is statistically significant, with a 0.0001 P value. The R square of the model is 0.1839.

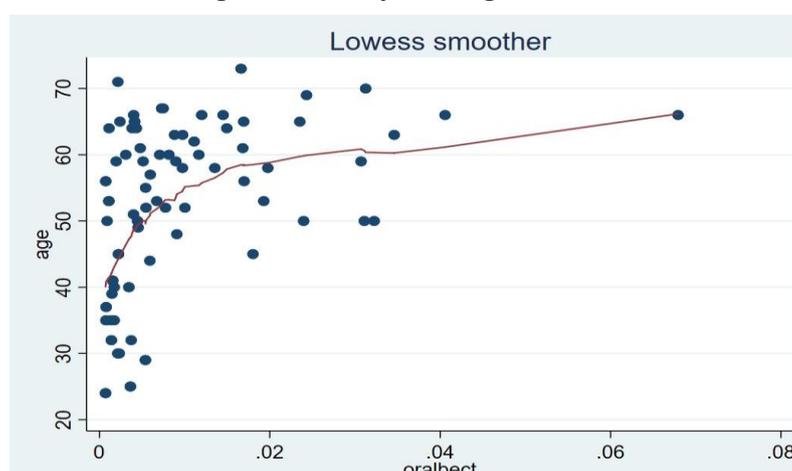
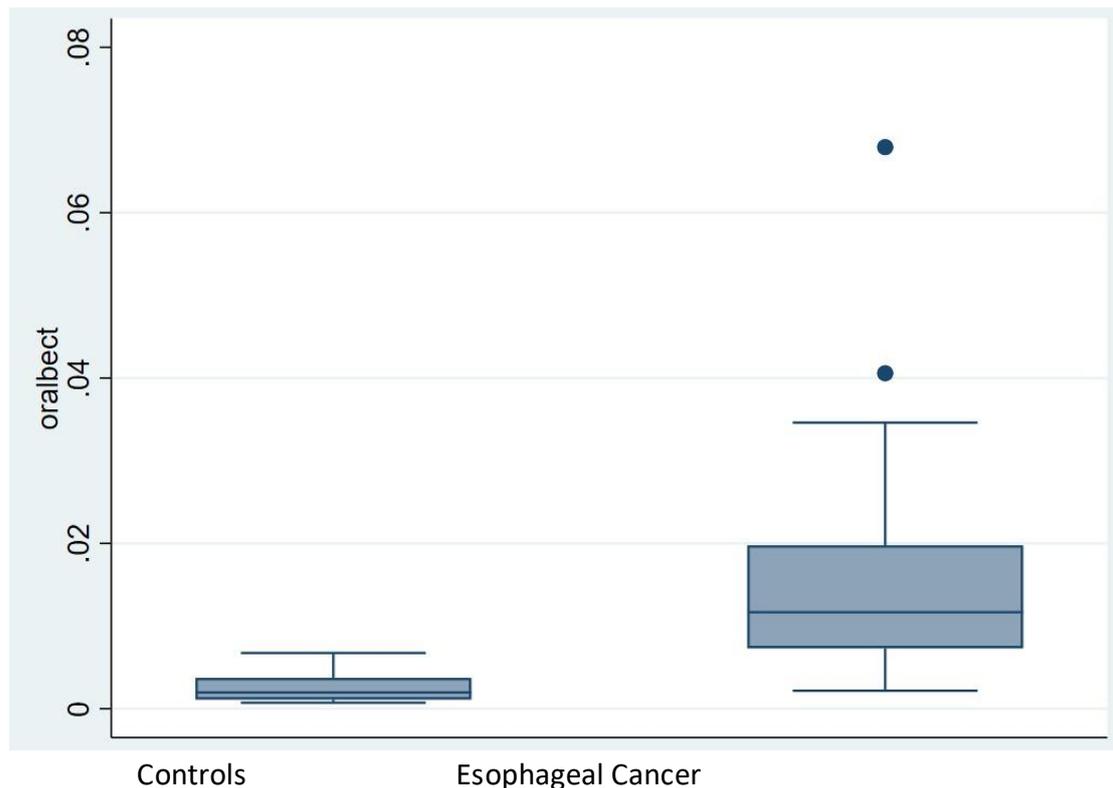
Figure 1: Scatter Regression Graph on Age with Concentration of Dental Porphyromonas

Table 2: Linear Regression on Age with Concentration of Dental Porphyromonas

Age	Coefficient	Standard Error	t	95% CI
Concentration on Dental Porphyromonas	513.86	118.09	4.35	(279.02, 748.70)
P Value	0.001			
R Square	0.1839			

The major study objective is to find out whether has associations between Dental Porphyromonas Concentration with Esophageal Cancer. From the boxplot graph in Figure 2 we can see that the median of Dental Porphyromonas Concentration is much higher in Esophageal Cancer group, compare with controls. The 75% quintile and maximum range are also much greater in esophageal cancer group compare with control group.

Figure 2: Boxplot of Concentration on Dental Porphyromonas between Esophageal Cancer and Controls



Based on the previous boxplot result we performed a two-sample t-test on mean concentration Dental Porphyromonas difference, between Esophageal cancer group and healthy controls. From Table 3, result showing that the mean concentration on Dental Porphyromonas in Esophageal cancer group is 0.0157, with standard error (SE) of 0.0019, while the mean and SE in control groups are 0.0024 and 0.0003. The

difference of mean between groups is 0.0132, with a P value of 0.0001, showing statistically significant of the result. Converting the result into multiple format, the mean of Dental Porphyromonas concentration from Esophageal Cancer group, are 6.54 times higher than Healthy Controls.

Table 3: Comparison on Dental Porphyromonas Concentration between Esophageal Cancer Group with Healthy Control.

Concentration on Dental Porphyromonas	Esophageal Cancer (n=43)	Control (n=1745)
Mean (SE)	0.0157 (0.0019)	0.0024 (0.0003)
95% Confidence Interval	(0.0118, 0.0196)	(0.0019, 0.0030)
Mean Difference (SE)	0.0132 (0.0019)	
t value	6.794	
P value	0.0001	

Our Major study results are presenting from Table 4. Dental Porphyromonas are found to have 1.23 odds higher than control, on developing Esophageal Cancer, with a marginal P value 0.046.

We also noticed that Guangdong population are keen to have hot tea drinking habit, From Table 4 we can see that, Hot tea drinking are having a 1.33 OR on Esophageal Cancer, with statistically significant.

Smoking also should be a classical risk factor on Esophageal cancer, with a 1.13 Odds ratio.

Table 4: Multi-variate Regression Result (Odds Ratio)

	Esophageal Cancer (n=43)	Control Group (n=1853)	Odds Ratio (OR)	P value
Dental Porphyromonas			1.23	*0.046
High Expose (n=261)	7 (2.7%)	254 (97.3%)		
Normal (n=1635)	36 (2.1%)	1599 (97.9%)		
Oral Infection Status			1.02	0.21
Yes (n=260)	6 (22.2%)	254 (18.3%)		
No (n=1636)	37 (77.8%)	1599 (81.7%)		
Current Smoking Status			1.13	*0.048
Yes (n=1274)	30 (65.9%)	1244 (67.5%)		
No (n=622)	13 (34.1%)	609 (32.5%)		
Daily Alcohol using			1.30	0.37

Yes (n=761)	20 (45.9%)	741 (39.2%)		
No (n=1135)	23 (54.1%)	1112 (60.8%)		
Hot Tea Drinking Habit			1.33	*0.02
Yes (n=546)	15 (33.7%)	531 (28.0%)		
No (n= 1350)	28 (66.3%)	1322 (73.0%)		

All results were adjusted with age, gender, and * with significant under 0.05 P cut off.

Discussion:

Bacteria and esophageal cancer:

As early as 1982, Finlay used microbial culture methods to show that normal people's esophagus was a sterile environment, or just "passing" bacteria. These "passing" bacteria are acquired either by swallowing or by gastroesophageal reflux [23]. Since then, Gagliardi and Pei et al. found that the number of streptococcus in the pharyngeal and esophageal mucosal tissues of the normal population was the highest, followed by prevotella (17%) and veronococcus (14%) [24]. Norder Grusell et al. also proved that the esophagus has its own microflora, which is very similar to the oral microflora, and the most common bacterial species is streptococcus aeruginosa.

In recent years, due to the development and application of high-throughput gene chip and second-generation sequencing technology, more than 700 kinds of bacteria that could not be cultured in the past have been recognized, and more than 300 kinds of oral microbial communities have been recognized (only 300 can be found in ordinary culture). In the study of bacteria in the esophagus, Zhiheng Pei et al. used 16s rDNA PCR technology to detect and found that the most common bacteria in normal distal esophageal tissue were streptococcus, platycoccus and veronococcus. Yang et al. used the same sequencing technology to detect the microflora in the distal esophagus of healthy people, esophagitis and Barrett's esophagus, and found that the normal esophagus was dominated by streptococcus, while the esophagitis and BE esophagus were dominated by gram-negative anaerobe [5]. However, the changes of bacterial flora in esophageal cancer tissues such as esophageal squamous cell carcinoma have not been reported.

There are many similarities between the oral microbiota and the esophageal microbiota, as most of the esophageal microbiota originate from the oral cavity. At present, studies on the relationship between salivary bacteria and esophageal cancer and its application value in predictive diagnosis have not been reported at home and abroad. Shantou city in Guangdong province is a region with high incidence of esophageal squamous cell carcinoma. We believe that since saliva is swallowed through esophagus into stomach, the stomach is a highly acidic environment and is not suitable for bacterial growth. In patients with esophageal cancer, due to esophageal dysplasia or tumor blockage of the esophagus, salivary bacteria stay in the local esophagus or esophageal cancer tissue for a long time and self-colonize, then enter the mouth with reflux fluid.

From our definitive study on Dental Porphyromonas Concentrations with Esophageal Cancer, we found out the mean of Dental Porphyromonas concentration from Esophageal Cancer group, are 6.54 times higher than Healthy Controls, with statistically significant, which making a further step to providing evidence on the association between oral bacteria with esophageal cancer.

Strength and Limitations of the study:

Although we are conducting the relatively reliable prospective cohort study, the exposures are yet to be clearly defined as a truly causal relationship due to the limitation on Guangdong population and its sample size.

Still, when comparing with the existing researches, especially those with retrospective design, we do have some strengths. Recall bias should be one of the limitations on the retrospective study. Comparing with the subjects without a certain condition, disease patients tend to recall of multiple potential harmful habits and status when they asked if these are related with their disease. Although those factors can be only a confounder of the situation.

Proof of temporality on disease outcome and exposures. In this study, we found out that there have associations between Dental Porphyromonas Concentration with Esophageal Cancer, and this evidence is partial sufficient to proof there have a causal relationship. Because the exposures are tent to happen before the outcomes.

Conclusion:

From this study, we found that Dental Porphyromonas are associated with Esophageal Cancer with a 1.23 odds ratio. Also, age might be a potential confounder of the result, which showing positive linear associated with Dental Porphyromonas, although the 0.1839 of R square are showing relatively low compliance on the model. Hot Tea

Drinking habit showing significant result on relationship with Esophageal Cancer, with a 1.33 Odds ratio, as well as current smoking status.

Further broader populational longitudinal study are needed to conduct, in order to clarify the causal relationship between Dental Porphyromonas Concentration and Esophageal Cancer, as well as minimizing confounders.

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