

Association between Upper Digestive Tract Microbiota and Cancer-Predisposing States in the Esophagus and Stomach

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Abstract

Background: The human upper digestive tract microbial community (microbiota) is not well characterized and few studies have explored how it relates to human health. We examined the relationship between upper digestive tract microbiota and two cancer-predisposing states, serum pepsinogen I/pepsinogen II ratio (PGI/II; predictor of gastric cancer risk) and esophageal squamous dysplasia (ESD; the precursor lesion of esophageal squamous cell carcinoma; ESCC) in a cross-sectional design.

Methods: The Human Oral Microbe Identification Microarray was used to test for the presence of 272 bacterial species in 333 upper digestive tract samples from a Chinese cancer screening cohort. Serum PGI and PGII were determined by ELISA. ESD was determined by chromoendoscopy with biopsy.

Results: Lower microbial richness (number of bacterial genera per sample) was significantly associated with lower PGI/II ratio ($P = 0.034$) and the presence of ESD ($P = 0.018$). We conducted principal component (PC) analysis on a β -diversity matrix (pairwise difference in microbiota), and observed significant correlations between PC1, PC3, and PGI/II ($P = 0.004$ and 0.009 , respectively), and between PC1 and ESD ($P = 0.003$).

Conclusions: Lower microbial richness in upper digestive tract was independently associated with both cancer-predisposing states in the esophagus and stomach (presence of ESD and lower PGI/II).

Impact: These novel findings suggest that the upper digestive tract microbiota may play a role in the etiology of chronic atrophic gastritis and ESD, and therefore in the development of gastric and esophageal cancers. *Cancer Epidemiol Biomarkers Prev*; 23(5); 735–41. ©2014 AACR.

Introduction

The human digestive tract harbors diverse microbial organisms. These microbial organisms collectively compose a microbial community called the microbiota. Microbial organisms in the gut interact with each other and with the host immune system in ways that influence the development of disease (1). Imbalances in the composition of gut microorganisms and induced changes in their inter-

action with the human host have been associated with the development of obesity, diabetes, and other diseases (2–6). *Helicobacter pylori* is the only bacterial species that is known to thrive in the acidic environment of the normal stomach. Inflammatory responses to *H. pylori*, however, sometimes result in the destruction of the cells that secrete acid into the stomach, leading to a condition known as chronic atrophic gastritis. This condition results in a neutralized stomach environment that, in turn, can be colonized by many other bacterial species, and this new colonization may play an important role in gastric carcinogenesis (7). However, the relationship between the whole microbiota of the upper digestive tract, chronic atrophic gastritis, and gastric cancer has not yet been established.

Pepsinogen I (PGI) and pepsinogen II (PGII) are aspartic proteinases that can hydrolyze peptides in acid environments (8). Some of the secreted molecules are released into the circulation and can be measured in serum. A low serum PGI/II ratio correlates well with the extent of chronic atrophic gastritis, and has been consistently related to gastric cancer (9). Indeed, the PGI/II ratio has been proposed as a screening test for both chronic atrophic gastritis and gastric cancer (10–12).

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Esophageal squamous dysplasia (ESD) is the precursor lesion of esophageal squamous cell carcinoma (ESCC). Some previous studies suggest that chronic atrophic gastritis, defined by a low serum PGI/II ratio, is associated with the risk of both ESD and ESCC (13–16). A study using the same Chinese cohort that is evaluated in the current analysis also showed that lower serum PGI/II was associated with increased risk of ESD (17), but a prospective study in the same population failed to find such an association with ESCC (9).

In the current analysis, we investigated the relationship between the upper digestive tract microbial richness and β -diversity (pairwise difference in microbiota among samples), serum PGI/II ratio and ESD in the Cytology Sampling Study 2 (CSS2), a cancer screening study in Linxian, China, a region with very high rates of ESCC and gastric cancer.

Materials and Methods

The study subjects, determination of ESD status, and serologic assays

The subjects were enrolled from three villages in Linxian, Henan Province, China in the spring of 2002. A description of the parent study, the CSS2, has been published previously (18). Subjects were volunteers, ages 40 to 65 years, apparently healthy, and had no contraindications for endoscopy (19). In total, 720 subjects were recruited for CSS2. Within the cohort, 659 had good quality microbiota data by Human Oral Microbe Identification Microarray (HOMIM); 375 had previously been selected for the measurement of serum PGI and PGII. The subset with PGI and PGII data were cases diagnosed with moderate or severe dysplasia in the biopsies of the squamous esophagus and the two sex-matched controls with normal biopsies. The details of diagnosis were described in the previous publication (19). In our study, 333 subjects with both PGI and PGII and microbiota data were analyzed, including 142 with ESD and 191 without ESD. ESD status was determined by chromoendoscopy with biopsy. Serum PGI and PGII were measured by ELISA (Biohit ELISA Kit, Finland). The details were described previously (18–20).

Sample collection and DNA extraction

Upper digestive tract cell samples were collected by one of the two devices (randomly allocated). One was an inflatable rubber balloon covered with cotton mesh attached to a 0.2 cm diameter single lumen rubber tube (manufactured in China). The second was the Cytomesh Esophageal Cytology Device (Wilson-Cook Medical, Inc). Each volunteer was given 2 mL of 2% lidocaine slurry by mouth before balloon insertion, inflation, and withdrawal. The head of the balloon was cut off and deposited in 40 mL of sterile saline. The tube with the balloon head was vortex for 30 seconds, then the balloon head was removed, and the remaining fluid was centrifuged at 1,500 RPM for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 1 mL saline, frozen in liquid nitrogen,

and stored at -80°C . DNA was extracted by the Genra Puregene Cell Kit (Qiagen).

DNA preparation for the array hybridization and HOMIM array

The HOMIM, which can detect 272 known human microbial species, was conducted in the Paster laboratory and the details of the method were described previously (21). The array uses 16S rRNA-based oligonucleotide probes printed on glass slides. The extracted DNA was used as the template in PCR reactions with universal forward and reverse primers and labeled in a second nested PCR. After hybridization, the washed slides were scanned using GenePix Pro software. A normalized median intensity score was generated by subtracting the median feature intensity from the background intensity for each individual feature. The relative intensity of each probed species/strain was then estimated using feature-specific criteria to determine the presence/absence of the bacterial species (21).

For quality control, five samples, selected on the basis of the largest amount of DNA available, were measured once in each batch to assess technical replication of the array results. Our analysis showed consistent richness results between batches. Furthermore, for 10 subjects, we cloned and sequenced PCR products (range 59–89) to assess whether any high abundance species were present in the samples but missing from the array. Only one species was present in multiple samples but absent from the array (*Lachnospiraceae* OT107).

Statistical analysis

Because some probes could hybridize with more than one species, microbial richness was estimated as number of genera per sample. The accuracy of the bacterial richness estimation above the species level by microarrays has been validated by both quantitative PCR and 16S rRNA gene clone libraries (22, 23). Association analyses were completed using R (24). We had one outcome that was measured as a continuous response (PG I/II ratio) and one outcome measured as a dichotomous response (ESD), and there were some differences in association for PG I/II ratio when we accounted for ESD (see below). Therefore, we used a variety of models to explore these associations. The associations between serum PGI/II and microbial richness were evaluated by linear regression models using richness as the dependent variable because of the heterogeneous results by ESD status (microbial richness = PGI/II + age + gender + smoking + antibiotic usage within the last 3 months + balloon sampling device). The associations between ESD status and microbial richness were evaluated by logistic regression models (ESD = microbial richness + age + gender + smoking + antibiotic usage within the last 3 months + balloon sampling device). The microbial richness was standardized by dividing it by its SD.

Estimation of microbial β -diversity was measured by unweighted UniFrac distance matrix, which measures pairwise microbial difference among samples (25).

UniFrac estimation was performed in FastUniFrac (25). The phylogeny used for UniFrac estimation was constructed from the Human Oral Microbiome Database (26).

UniFrac distance was used in two ways. First, the unweighted UniFrac matrix was analyzed by principal component (PC) analyses. The first three principal components were used to summarize β -diversity. As noted above, the associations between serum PGI/II and each principal component were evaluated using linear regression models (PC = PGI/II + age + gender + smoking + antibiotic usage within the last 3 months + balloon sampling device). The associations between ESD status and each principal component were evaluated using logistic regression models (ESD = PC + age + gender + smoking + antibiotic usage within the last 3 months + balloon sampling device). Each principal component was standardized by dividing it by its SD. Second, all of the samples were clustered into groups by the UniFrac distance matrix based on unsupervised clustering algorithm (the unweighted pair group method with arithmetic mean (27) and then PGI/II or ESD status was regressed on these clusters and other factors.

To examine the association between PGI/II, ESD, and the presence/absence of individual strain/species targeted by each probe, we regressed PGI/II or ESD against the presence/absence of individual strain/species by logistic regression model.

Results

Characteristics of the study subjects

A description of the subjects evaluated in this study was provided in Table 1. Subjects with ESD had significantly lower PGI/II levels than subjects without this cancer-predisposing condition ($P = 0.044$, data not shown).

Significant association between number of genera per sample, a measure of microbial richness, and both the PGI/II ratio, and a diagnosis of ESD

In analyses adjusted for gender, smoking, age, antibiotic usage, and balloon sampling device, PGI/II was significantly positively associated with number of genera per sample ($P = 0.034$; Fig. 1A; Table 2). The magnitude of the association was little changed after further adjusted for ESD ($P = 0.059$; Table 2). However, when we also included an interaction between PGI/II and ESD, we found that it was statistically significant ($P = 0.029$), a reflection of the fact that the association between richness and PGI/II was significantly stronger in subjects with ESD compared with those without ESD. Therefore, we examined the association in separate models for those with and without ESD (Fig. 1B and C). Note the much steeper slope among subjects with ESD.

In analyses adjusted for gender, smoking, age, antibiotics usage, and balloon sampling device, the odds of ESD were decreased by a factor of 0.74 per one SD increase in microbial richness ($P = 0.018$; Table 3 and Fig. 2A). Similar results were found with additional adjustment for PGI/II ratio (OR = 0.76, $P = 0.032$). However, the association was

Table 1. Characteristics of the studied subjects from the CSS2 cancer screening study

	N (total = 333)	Percentage
Tobacco smoker		
Yes	42	12.6
No	291	87.4
Gender		
Female	162	48.6
Male	171	51.4
Balloon sampling device		
Chinese balloon	161	48.3
Wilson-Cook balloon	172	51.7
Antibiotic usage within the last 3 months		
Yes	42	12.6
No	291	87.4
Histologically diagnosed ESD		
Yes	142	42.6
No	191	57.4
	Mean	SD
Age	55.03	4.54
PGI/II ratio	9.40	4.12
Number of genera	22.93	6.11
Number of families	16.93	3.26
Number of orders	12.12	1.84
Number of classes	10.02	1.19
Number of phyla	5.37	0.72

found in those whose PGI/II ratio is below the median, as is seen by comparing results stratified by PGI/II ratio in Table 3 and Fig. 2B and C. Thus, the association of low risk with increasing number of genera is mainly in those who are more likely to have chronic atrophic gastritis.

We observed no association between microbial richness and antibiotic usage although previous studies have shown the strong association between antibiotic usage and change of microbial richness in fecal samples (28, 29). This may be due to our limited number of subjects with recent antibiotic usage (antibiotic usage in the last 3 months, $n = 42$). It is also possible that upper digestive tract microbiota recovers quickly from antibiotic treatment, as has been seen in fecal microbiota one week after treatment (28).

Significant association between microbial β -diversity and PGI/II, ESD status

To assess the associations between β -diversity and either PGI/II ratio or risk of ESD, we used the first three standardized principal components of the unweighted UniFrac distance matrix. PGI/II was significantly associated with PC1 and PC3 (Table 2), and these associations were independent of and did not interact with ESD (PC1, $P_{\text{interaction}} = 0.081$; PC2, $P_{\text{interaction}} = 0.833$). These analyses suggest that two independent aspects of

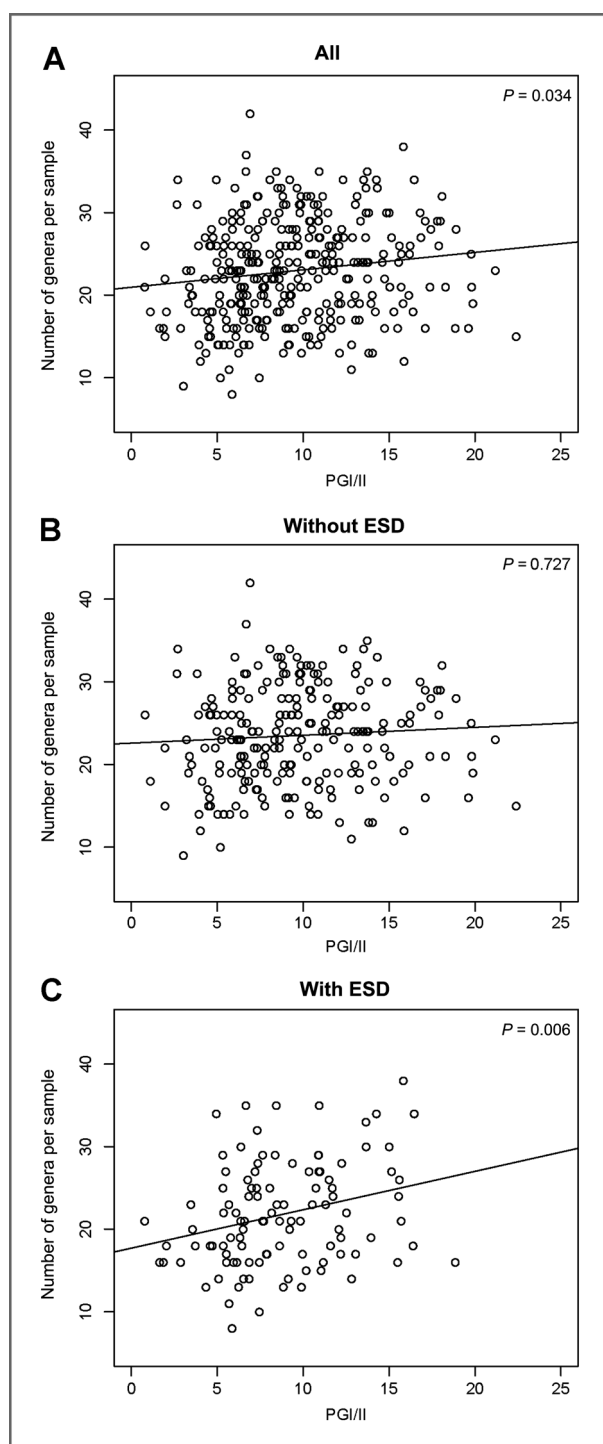


Figure 1. Plots of serum PGI/II and number of genera per sample in all studied subjects (A), subjects without ESD (B), and subjects with ESD (C). The regression lines and *P* values were from the adjusted linear regression models.

β -diversity were associated with the PGI/II ratio because principal components are designed to be uncorrelated. In contrast, only PC1 was associated with risk of ESD (Table 3). Of note, the magnitude of the association

between PC1, PC3, and PGI/II were not changed by adjustment for ESD (Table 2), and the magnitude of the association between PC1 and ESD was hardly changed by adjustment for PGI/II (Table 3).

Subjects were grouped into three clusters based on the similarities of their microbial communities, as assessed by UniFrac distances. We compared the PGI/II ratio and prevalence of ESD among subjects in the two large clusters, which are labeled B and C in Supplementary Fig. S1. The odds of ESD were 1.41-fold higher in cluster C than in cluster B after adjustment for smoking, gender, age, balloon sampling device, and antibiotic usage in the last 3 months [95% confidence interval (CI) for OR: 1.12–1.79; *P* = 0.004], and results were little changed after further adjustment for PGI/II ratio (OR and 95% CI: 1.42 (1.12–1.80); *P* = 0.004). We found no difference in PGI/II ratio between subjects in cluster B and C (*P* = 0.766).

No significant association between PGI/II, ESD, and presence/absence of specific targeted species/strain

Supplementary Tables S1 and S2 show no association between the presence/absence of each targeted species/strain and PGI/II ratio or ESD status after Bonferroni correction for multiple comparisons.

Discussion

In this cross-sectional study, we found a significant positive association between microbial richness and PGI/II ratio, and an inverse association between microbial richness and ESD. Because low PGI/II ratio is a marker for chronic atrophic gastritis, our results suggest that individuals with lower microbial richness were more likely to have chronic atrophic gastritis and ESD. We also observed evidence for a statistical interaction between microbial richness and these two cancer-predisposing states. The association between microbial richness and PGI/II ratio was significantly stronger in subjects with ESD. Similarly, the association between microbial richness and ESD was stronger in those with a lower PGI/II ratio. Although the number of genera, a measure of microbial richness, measures a property of an individual, β -diversity measures the similarity between microbiota in pairs of individuals. We also found associations between risk of ESD, PGI/II ratio, and features of β -diversity.

Decreased microbial richness has been shown to be associated with disease states such as obesity, diabetes, inflammatory bowel disease, and others (3, 4, 6). Our findings are in line with these previous results. We found that low microbial richness in the upper digestive tract was associated with cancer-predisposing conditions of the stomach and esophagus. However, as in these prior cross-sectional studies, our cross-sectional data cannot distinguish whether decreased microbial richness causes these cancer-predisposing states or is an effect of them. Our findings strongly suggest the need

Table 2. Associations between upper digestive tract microbial richness or β -diversity metrics and PGI/II ratio in the CSS2

	Adjusted model ^a		Model further adjusted for ESD ^b		Interaction ESD*PGI/II ^b
	Coefficient	P	Coefficient	P	P
Microbial richness					
Number of genera	0.029 ± 0.013	0.034	0.025 ± 0.013	0.059	0.029
β -diversity					
PC1	-0.038 ± 0.013	0.004	-0.035 ± 0.013	0.009	0.081
PC2	0.013 ± 0.014	0.346	0.015 ± 0.014	0.287	0.904
PC3	0.034 ± 0.013	0.009	0.034 ± 0.013	0.011	0.833

NOTE: Bold indicates *P* value less than 0.05.^aLinear regression model adjusted for smoking, gender, age, balloon sampling device, and antibiotic usage in the last 3 months.^bLinear regression model further adjusted for ESD besides smoking, gender, age, balloon sampling device, and antibiotic usage in the last 3 months.

for prospective studies of the upper digestive tract microbiota and the development of gastric cancer and ESCC.

The normal acidic stomach is known for its paucity of bacterial genera. *H. pylori* is usually the only bacterial species that can grow in the stomach. Subjects with a low PGI/II ratio, however, are at high risk for chronic atrophic gastritis, a condition in which the stomach can no longer secrete normal amounts of acid. We would expect that a less acid stomach environment would allow more bacterial taxa to live, so that one would predict an inverse association between gastric microbial richness and PGI/II ratio. In our study, however, we found the opposite result, a positive association between upper digestive tract microbial richness and PGI/II ratio. One possibility is that alterations in the stomach due to chronic atrophic gastritis may result in overgrowth of few taxa and extinction of others, leading to the reduction of overall microbial richness. It should

be remembered, however, that our biologic sample included cellular and luminal material from the esophagus and oral cavity, as well as the stomach, which complicates our interpretation. Further investigation is required to clarify our results.

Compared with subjects without ESD, subjects with ESD had a significantly lower number of genera in the upper digestive tract. This may be because ESD causes changes in the local habitat (the surface of the esophagus and the esophagus mucosa), which might lead to extinction of certain taxa in the communities associated with the normal esophagus and/or overgrowth of other taxa. Alternatively, other risk factors for ESD, such as poor oral health, may be driving changes in the upper digestive tract microbiota.

The inverse association of microbial richness with the risk of ESD was stronger in subjects with low PGI/II ratios, and the positive association of microbial richness with PGI/II ratio was stronger in subjects with

Table 3. OR for ESD (and 95% CI) from logistic regressions on standardized upper digestive tract microbial richness or on standardized β -diversity metrics

	All (333)				Stratified by PGI/II ^a			
	Adjusted model ^a		Model further adjusted for PGI/II ^b		PGI/II < median		PGI/II ≥ median	
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
Microbial richness								
Number of genera	0.74 (0.58–0.95)	0.018	0.76 (0.59–0.97)	0.032	0.63 (0.43–0.89)	0.010	0.95 (0.66–1.37)	0.785
β -diversity								
PC1	1.45 (1.14–1.85)	0.003	1.41 (1.10–1.80)	0.006	1.63 (1.16–2.32)	0.005	1.19 (0.83–1.69)	0.350
PC2	1.13 (0.90–1.44)	0.291	1.15 (0.91–1.45)	0.238	1.27 (0.91–1.78)	0.166	1.06 (0.74–1.52)	0.758
PC3	0.94 (0.74–1.20)	0.634	0.97 (0.76–1.25)	0.847	1.01 (0.73–1.41)	0.939	0.93 (0.66–1.34)	0.701

NOTE: Bold indicates *P* value less than 0.05.^aModel adjusted for smoking, gender, age, balloon sampling device, and antibiotic usage in the last 3 months.^bModel adjusted for PGI/II besides smoking, gender, age, balloon sampling device, and antibiotic usage in the last 3 months.

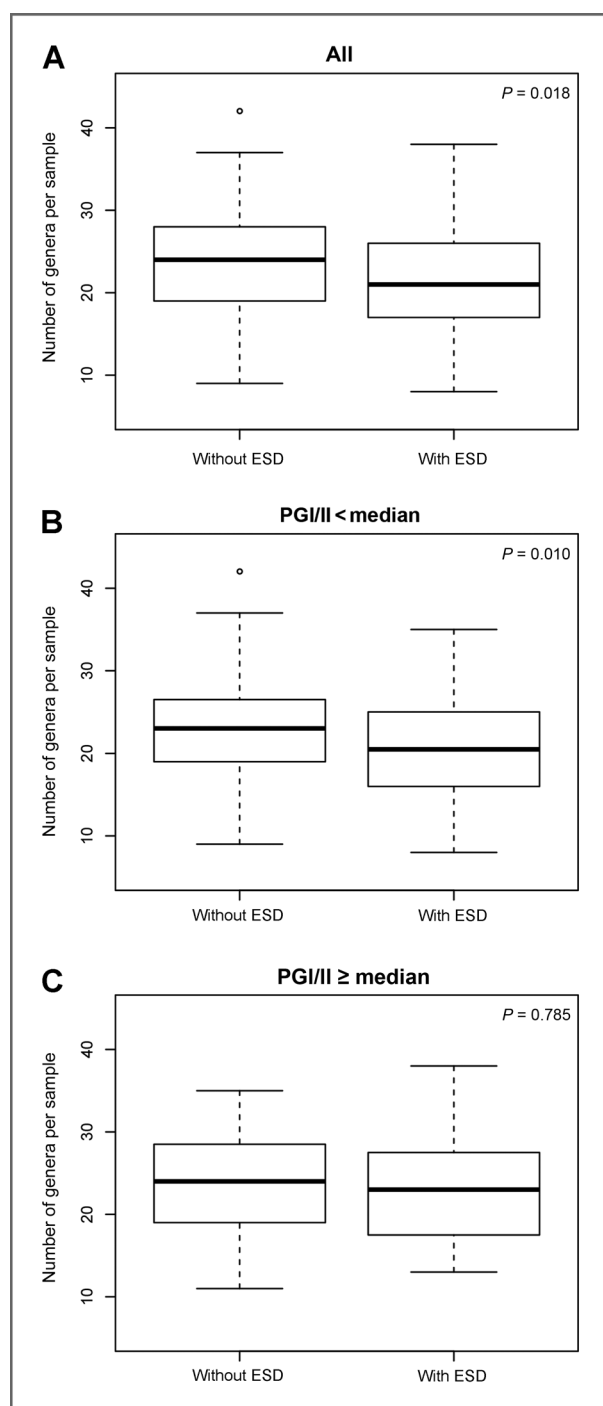


Figure 2. Boxplots of ESD status and number of genera per sample in all studied subjects (A), subjects with PGI/II less than median (B), and subjects with PGI/II equal to or greater than median (C). Boxes encompass the interquartile range (IQR), with central bars for medians and whiskers for 1.5 times the IQR. Outlier values are circles. P values were from the adjusted logistic regression models.

ESD. Lower PGI/II ratios are associated with increased prevalence of ESD (17). However, the mechanisms by which ESD might modulate the association of richness

with PGI/II or by which PGI/II might modulate the association of richness with ESD are unknown.

Our other important and connected finding was the significant association between β -diversity and both PGI/II and ESD. Our results suggest that these health conditions did contribute to the differences in upper digestive tract microbial communities observed between subjects. Previous studies of the gut microbiota have shown that diet, race, and geography contribute to the taxonomic similarity between two samples (30–32). Similarly, our findings showed that the similarity of upper digestive tract microbial communities between two individuals is associated with their upper digestive tract health status.

Our study has several strengths and limitations. The strengths include the relatively large sample size, the inclusion of asymptomatic healthy subjects, and the availability of detailed clinical and demographic information on participants. The limitations include the cross-sectional design of the parent study which precludes separating cause and effect. The limitations also include the use of an array designed primarily to assess the oral microbiota, and the use a sampling device that collected cellular and luminal material from the stomach, esophagus, and oral cavity. In addition, our study was conducted in a region of China with high rates of ESCC and gastric cardia adenocarcinoma. As seen previously, the risk factors in this region may differ from Western countries (33). Therefore, our result may not generalize to other populations.

Our results suggest that the microbiota in upper digestive tract may play an important role in the development of gastric and esophageal cancer. Monitoring or even modifying our upper digestive tract microbiota might provide an opportunity for predicting or even changing the risk of gastric and esophageal cancer. However, more investigation in prospective cohorts of people is needed to confirm whether and how the upper digestive tract microbiota may affect the development of gastric and esophageal cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. Yu, M. Gail, J. Shi, V. Klepac-Ceraj, G.-Q. Wang, J.-H. Fan, N.D. Freedman, C. Abnet

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Study supervision: W.-Q. Wei, Y.-L. Qiao, C. Abnet

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