Long-read sequencing revealed alterations of microbial relationship between tongue coating and gastric mucosa in patients with gastric intestinal metaplasia

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Abstract

Abnormalities of microbiota in tongue coating (TC) are associated with gastric cancer, however, the correlation between microbiota colonizing in TC and in gastric mucosa (GM) and their roles in the development of gastric cancer remain poorly understood. In this study, using long-read sequencing, we profiled the microbiota in both TC and GM from 44 patients with the precancerous gastric intestinal metaplasia (GIM) and 28 matched controls who were rapid urease test (RUT)-negative and had with non-symptomatic chronic superficial gastritis. While no significant difference in diversity of microbiota in either TC or GM was observed between GIM patients and the controls, the distribution of bacteria (operational taxonomic units, OTUs) shared by TC and GM was significantly different between GIM patients and the controls as well as between RUT-positive and RUT-negative GIM patients. LEfSe (Linear discriminant analysis effect size) identified TC Prevotella melaninogenica and three GM Helicobacter species (i.e., H. pylori, H. pylori XZ274, and H. pylori 83) that were enriched in GIM patients, suggesting a potential role of Hp infection in the development of GIM. In RUT-negative GIM patients, gastric Veillonella, Pseudonocardia, and Mesorhizobium were enriched. The commensal network between TC and GM was more complex in patients with GIM than that in controls, and more closely correlated in RUT-positive than in RUT-negative GIM patients. Consistent with the known contribution of H. pylori to lower values of PG-I/PG-II, the serum ratio of PG-I to PG-II was found negatively correlated with the three gastric Helicobacter species (H. pylori, H. pylori XZ274, and H. pylori 83) in RUT-negative GIM patients and negatively correlated with two TC species (Fusobacterium nucleatum subsp. nucleatum and Campylobacter showae) in RUT-positive GIM patients. In summary, the oral and gastric commensal linkage as well as H. pylori infection were promoted in GIM.

Novelty & Impact Statements

Gastric intestinal metaplasia (GIM) is a well-recognized precancerous lesion and is an independent risk factor for gastric cancer. The Oral-Gut microbiome axis was proved in many systemic disorders. Several evidences presented tongue coating (TC) microbiota had diagnostic value and reflected the development of gastric diseases, and the latest image-based AI deep learning diagnostic models presented the value of tongue images and tongue coating (TC) microbiome in the diagnosis of gastric cancer. This study explored the microbial linkage between TC and gastric mucosa (GM) in patients with GIM, and the present data showed that development of GIM promoted the commensal linkage between tongue coating and stomach, and H. pylori infection increased the bacterial correlation between tongue and stomach.

Introduction

Gastric intestinal metaplasia (GIM) is characterized by the chronic inflammation of gastric mucosa (GM), inherent gland atrophy, and the replacement of gastric epithelial cells with intestinal cells, such as Paneth's cells, goblet cells, and enterocytes [1]. GIM is a well-recognized precancerous lesion and is an independent risk factor for gastric cancer (GC), but its etiology remains unclear. Evidence showed that the incidence of GIM has important regional differences and that the proportion of GIM progression to GC
also varies dramatically [2]. These geographic differences may be attributed to different diagnostic criteria, genetics, lifestyle, and biological factors [3]. Thus, GIM has been considered as a combination of multiple factors, including *Helicobacter pylori* (Hp) infection, bile reflux, genetics, dietary habits, and stomach microbiota. [4]. With the development of culture-independent high-throughput sequencing technology, evidence suggested that abnormal gastric microbiota especially interaction between Hp and other bacteria are a critical factor leading to GM lesions besides GIM [5]. Our small-scale pilot study presents evident alteration of gastric bacteria in patients with GC in cancer tissues and surrounding noncancerous tissues [6]. Hp infection occurs and obviously promotes the colonization of oral tongue coating (TC) microbiota in the GM [7]. These results suggested that colonized oral microbiota in GM are likely involved in GM inflammation, leading to superficial gastritis, atrophic gastritis, intestinal metaplasia, and eventually gastric carcinoma.

The oral cavity is coated with a plethora of bacteria. Approximately $10^{11}$ oral bacteria enter the digestive tract through the swallowed saliva every day. The functional heterogeneity of different sections of the gastrointestinal tract leads to dramatic differences in microbiota [8]. Rashidi et al. analyzed the structure of saliva and fecal microbiota in healthy adults and found that salivary and fecal microbiota have little in common except for *Dialister invisus*, indicating that oral bacteria normally cannot survive through the gastrointestinal tract [9]. The mouth and stomach are connected by esophagus, and Wu et al. reviewed the relationship between TC microbiota and its potential diagnostic applications in many systemic diseases besides digestive disorders [10]. Cui et al [11] found TC/GM sharing *Campylobacter concisus* as a potential biomarker for gastritis including precancerous cascade, suggesting TC microbiota cross talking with gastric microbiota. In recent years, more and more evidences based on culture-independent high-throughput sequencing presented that non-Hp microbiota in the stomach has a potential role in the GC development of gastric cancer [12], Knippel et al believed that oral microbiota, tooth abscess are related to the GC risk, and the co-infection of Hp and EBV could promote the occurrence of Hp related GC [13]. Sun et al investigated the alteration of gastric microbiota from superficial gastritis to gastric cancer in a Hp negative population [14]. Li et al designed a prospective multicenter clinical cohort study and developed an AI deep learning tool for GC diagnostic, and found that both tongue images and the TC microbiota could be used as tools for the diagnosis of GC [15]. These results strongly indicated that the interacting oral-stomach microbiota link the development of gastric disorders, however, the relationship between oral microbiota and gastric microbiota is still poorly understood.

Thus, this study focuses on the oral TC and gastric microbiota and aims to explore the effects of Hp infection on the bacterial crosstalk between TC and GM microbiota when GIM occurs. Results showed that GIM and Hp infection considerably affect the colonization of TC microbiota in GM and that Hp infection negatively affects the colonization of TC microbiota in GM. These results add a new understanding of linkage between oral and gastric microbiota involved in gastric precancerous lesions at least in GIM.

**Material And Methods**
Study Population

The project was approved by the Ethics Committee of People's Hospital of Yangzhong City (No. 2018-039), and all patients had been informed and retained the signatures. From April 2019 to January 2020, 44 patients with GIM diagnosed by gastroscopy and pathology were recruited, and 28 volunteers diagnosed with chronic superficial gastritis without any clinical symptom (e.g., stomach pain, bloating, abdominal pain, diarrhea, and bile reflux) were chosen as control group. A unified questionnaire was used to collect clinical data. The exclusion criteria were: 1) history of major organic diseases, such as stroke, malignant tumors, and organ transplantation; 2) history of antibiotics, probiotics, proton pump inhibition, and other drugs within the passing one month; 3) over 70 years old; 4) less than 5 years of residence time in Yangzhong city; and 5) unwilling to cooperate. In particular, there were no patients with Corona Virus Disease 2019 (COVID-19) enrolled in this study because of the strict measures to block virus transmission.

Tongue Coating And Gastric Mucosa Sampling

All participants fasted for at least 8 h. TC samples were collected by disposable sterile toothbrush in the morning as described previously [16]. GM samples were collected in accordance with the published literature [17]. Considering that the pathological change was unpredictable, double-portion sampling was conducted for endoscopic and histological examinations. The tissue samples of GM were obtained by standard gastroscopic forceps, the GM samples for pathological examination were fixed in 10% formalin, and the GM samples for microbial analysis were frozen in liquid nitrogen immediately and stored at −80°C within three months. The GIM presented differentiated epithelium in the area of the glands and superficial epithelium. The controls had a negative result of rapid urease test (RUT) and no digestive symptom, such as stomachache, gastric distention, and abnormal feces. The patients with GIM were regarded as Hp infection with positive RUT, whereas the patients with GIM were regarded as non-Hp infection with negative RUT.

DNA Extraction And Microbial Sequencing

The total DNA was extracted from TC and GM by using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, United States), and the quality of total DNA was detected by 1% agarose gel electrophoresis. The V1-V9 region of the 16S rRNA gene was amplified using universal primers 27F (5'-AGRGTGATGMRBACTGCAG-3') and 1492R (5'-RGTYACCTTCTTACGACTT-3'). The conditions of polymerase chain reaction were as follows: initial denaturation at 98°C for 1 min; 27 cycles of 30 s at 95°C, 30 s at 55°C, and 45 s at 72°C; and final extension at 72°C for 10 min. Amplification products were isolated by 2% agarose gel electrophoresis and retrieved by AMPure XP beads (Beckman coulter GmbH, Krefeld, Germany). The purified amplification was ligated with a 6bp barcode unique to each sample by using the SMRTbell barcode Adapter Compete Prep-96 kit (Pacific Bioscience, Florida, United States). The bacterial library was constructed by the Pacific Biosciences SMRT bell™ Template Prep kit 1.0 (Pacific Bioscience,
Florida, United States) and profiled by the PacBio SMRT sequencing technology in accordance with a previous report [18]. Because of the low biomass of TC and GM, here introduced extraction negative control and PCR negative sample control to eliminate the possibility of extraneous contamination. The experiment entrusted Shanghai Biozeron Biotechnology Co., Ltd, whose technical analysis of high-throughput sequencing conforms to the GB/T 19001 – 2016/ISO9001:2015.

Data sequencing was proceeded as in previous publication [19]. First, raw data were filtered and optimized by the software package Mothur (version 1.17), and the Usearch 6.1.544 was used to cluster operational taxonomic units (OTUs) based on 97% similarity. OTUs were aligned to the Ribosomal Database Project II database, and each OTU was annotated to certain phylum, class, order, family, genus, and species. Bioinformatics analysis was conducted using the software Visual Genomics (Release 1.4.1, http://amplicon.vgenomics.cn/), including alpha diversity, community structure, linear discriminant analysis effect size (LEfSe). SparCC correlation analysis was used to construct the commensal network, and heatmaps were presented using R software (https://www.r-project.org/).

**Immunoblotting Assay Of Serum Hp-igg**

The Hp immunoblotting assay kit (No. 201905016, Shenzhen Blot Biological Products Co., Ltd, China) was used to detect serum antibodies against four Hp antigens, including cytotoxin-associated gene product (CagA, 116kD), vacuolating cytotoxins (VacA, 95kD & 91kD), and urease A (UreA, 30kD) and B (UreB, 66kD). Briefly, the Hp-blot strip was placed into the detection tank and added with 1000 µL diluent and 10 µL equilibrated serum. The mixture was shaken at 40 times/min and room temperature (22°C – 23°C) for 30 min, washed thrice with 1000 µL washing buffer, added with 500 µL working solution and 10 µL horseradish peroxidase (HRP) labeling antibodies, shaken at 40 times/min at room temperature (22°C – 23°C) for 30 min, and washed thrice with 1000 µL washing buffer. Finally, the mixture was added with 500 µL chromogenic solution, allowed to stand for 5 min, and washed thrice with 1000 µL washing buffer to terminate the reaction. The type of Hp was determined by the four antibodies. Pathogenic I type Hp was diagnosed by positive anti-UreB and anti-UreA, positive anti-CagA, and/or anti-VacA. Nonpathogenic II type Hp was diagnosed by positive anti-UreB and anti-UreA, negative anti-CagA, and anti-VacA.

**Statistical Analysis**

All statistical analyses were performed using SPSS (version 23.0, SPSS Inc., Chicago, IL, United States). The chi-square test was used for grade data, and nonparametric test or Student t-test was used for continuous data. All statistical analyses were two-sided tests, and \( P<0.05 \) was considered statistically significant.

**Results**

**General Clinical Characteristics**
A total of 44 patients with GIM and 28 healthy controls were enrolled in the study. The general clinical characteristics are presented in Table 1. No statistically significant difference in gender, smoking, drinking alcohol, drinking tea, and eating pickled food was observed between the two groups ($P > 0.05$). Patients with GIM were older than the controls ($P < 0.05$). The proportion of bile reflux in patients with GIM was significantly higher than that in controls ($P < 0.05$). The BMI, serum triglycerides, and PG-I/PG-II ratio of patients with GIM were significantly lower than those of the controls ($P < 0.05$). Although no significant difference in the serum Hp-Ig distribution was observed between the two groups ($P > 0.05$), the ratio of Hp-I in patients with GIM was significantly higher than that in controls ($P < 0.01$). Among the patients with GIM, 15 (34.1%), 31 (70.5%), 21 (47.7%), 17 (38.6%), 12 (27.3%), 20 (45.5%), 15 (37.4%), 21 (47.7%), 8 (18.2%), and 16 (36.4%) cases had family history of digestive tract cancers, chills, epigastralgia, stomach distension, abnormal stools, heart palpitations, insomnia or abnormal dreams, hot drink consumption habits, stomach pain relief with warmth, and halitosis, respectively. These data suggested that aging, bile reflux, and infection history of toxic Hp were the key contributors to GIM development.
The common clinical characteristics of the population

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls (n = 28)</th>
<th>GIM patients (n = 44)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (Male/Female)</td>
<td>12/16</td>
<td>21/23</td>
<td>0.686 a</td>
</tr>
<tr>
<td>Age (years)</td>
<td>54.2 ± 7.9</td>
<td>60.4 ± 6.4</td>
<td>&lt; 0.001 b</td>
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<td>BMI (kg/m²)</td>
<td>24.2 ± 2.5</td>
<td>22.8 ± 2.4</td>
<td>0.017 b</td>
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<tr>
<td>Smoking (Yes/No)</td>
<td>5/23</td>
<td>14/30</td>
<td>0.190 a</td>
</tr>
<tr>
<td>Drinking alcohol (Yes/No)</td>
<td>11/17</td>
<td>20/24</td>
<td>0.606 a</td>
</tr>
<tr>
<td>Drinking tea (Yes/No)</td>
<td>15/13</td>
<td>22/22</td>
<td>0.768 a</td>
</tr>
<tr>
<td>Eating pickled food (Yes/No)</td>
<td>25/3</td>
<td>43/1</td>
<td>0.127 a</td>
</tr>
<tr>
<td>RUT (Positive/Negative)</td>
<td>0/26</td>
<td>23/21</td>
<td>&lt; 0.001 a</td>
</tr>
<tr>
<td>Serum Hp-IgG (Positive/Negative)</td>
<td>24/4</td>
<td>42/2</td>
<td>0.145 a</td>
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<tr>
<td>Hp type (I/II)</td>
<td>12/12</td>
<td>35/7</td>
<td>0.006 a</td>
</tr>
<tr>
<td>Bile reflux (Positive/Negative)</td>
<td>0/28</td>
<td>3/35</td>
<td>0.011 a</td>
</tr>
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<td>Plasma glucose (mmol/L)</td>
<td>5.500(5.200,5.900)</td>
<td>5.350(5.100,5.700)</td>
<td>0.286 c</td>
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<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.040(4.180,6.205)</td>
<td>4.630(3.798,5.313)</td>
<td>0.093 c</td>
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<tr>
<td>Triglycerides (mmol/L)</td>
<td>2.210(1.563,2.660)</td>
<td>1.386(0.970,2.023)</td>
<td>0.001 c</td>
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<tr>
<td>LDL-C (mmol/L)</td>
<td>2.205(1.880,3.213)</td>
<td>2.230(1.780,2.465)</td>
<td>0.219 c</td>
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<tr>
<td>HDL-C (mmol/L)</td>
<td>1.460(1.178,1.700)</td>
<td>1.490(1.260,1.653)</td>
<td>0.922 c</td>
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<td>Pepsinogen (ng/mL)</td>
<td>48.10(37.33,59.92)</td>
<td>60.15(37.70,86.28)</td>
<td>0.139 c</td>
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<tr>
<td>PG I/II</td>
<td>4.645(4.073,5.798)</td>
<td>2.890(2.163,5.028)</td>
<td>0.004 c</td>
</tr>
</tbody>
</table>

LDL-C: Low Density Lipoprotein Cholesterol; HDL-C: High Density Lipoprotein Cholesterol. a Chi-square test, b Student-t test, and c Mann-Whitney U test.

Diversity And Community Structures Of Microbiota

In general, average 25901 ± 6503 and 25768 ± 6200 reads were obtained in the GM samples and TC samples, respectively. Alpha diversity analysis was conducted to assess the microbial richness and diversity, and three richness indices (Ace, Chao, and Observed OTUs) and two diversity indices (Shannon,
Simpson) were determined using the Mann–Whitney $U$ test. However, no significant difference in the diversity of TC and GM microbiota was observed between the two groups ($P > 0.05$, Table S1). The results indicated that the development of GIM have no effect on the microbial diversity in the oral cavity and stomach.

Then, here observed the microbial structure in the TC and GM. Based on the sequencing data, 275 OTUs were detected, which belong to eight phyla, 13 classes, 31 orders, 48 families, 78 genera, and 35 known species. The results of microbial structural analysis are presented in Fig. 1. The dominant phyla included Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Epsilonbacteriaeota, and Fusobacteria. Although the phylum composition was significantly different between TC and GM, no evident alteration was observed between patients with GIM and controls (Fig. 1a). The dominant bacterial genera of TC were Neisseria, Veillonella, Prevotella 7, Streptococcus, Porphyromonas, and Prevotella, whereas the dominant genera of GM were Ralstonia, Mesorhizobium, Pseudonocardia, Bradyrhizobium, Labrys, Pseudolabrys, Variovorax, Hydrobacter, Reyranella, Acidovorax, and Helicobacter (Fig. 1b). At the species level, Prevotella melaninogenica, Streptococcus infantis X, Veillonella atypica, and Neisseria subflava were the core bacteria in the dorsal surface of tongue, whereas Pseudonocardia sp. AL041005-10, Bradyrhizobium sp. BTAi1, and Hp 83 were dominant in gastric microbiota (Fig. 1c).

On the macro level, the community structural similarity of TC and GM microbiota was measured by the Jaccard distances at the OTU level. However, the difference between TC and GM was not statistically significant between the two groups ($P > 0.05$, Fig. 1d, Supplementary data for Fig. 1d). Subsequently, the number of co-occurring OTUs between paired TC and GM was defined as TC/GM sharing OTUs. Controls had an average of 24.4 TC/GM sharing OTUs (range: 4–81, Fig. 1e, Supplementary data for Fig. 1e), and patients with GIM had an average of 28.7 TC/GM sharing OTUs (range: 4–76, Fig. 1f, Supplementary data for Fig. 1f). The number of TC/GM sharing OTUs was divided into four grades: $\leq$ 20, 21–40, 41–60, and $\geq$ 60. Compared with that in controls, the percentage of TC/GM sharing OTUs in patients with GIM was significantly higher at 41–60 ($\chi^2 = 57.263$, $P < 0.001$; Fig. 1g, Supplementary data for Fig. 1g). These results showed increased sharing OTUs between the two groups despite the significantly different structure between TC and GM. The percentage of TC/GM sharing OTUs in patients with GIM was significantly higher than that in controls, suggesting that GIM promoted the similarity of the microbiota between TC and GM or the microbial migration from TC to GM.

Alteration Of Tc And Gm Microbiota In Patients With Gim

To observe the altered characteristics of the oral and gastric microbiota in the patients with GIM, differential microbiota was analyzed between patients with GIM and controls at the phylum, genus, and species levels. The microbiota with high and low relative abundance values were analyzed by the Mann–Whitney $U$ and Chi-square tests, respectively. A total of four phyla, nine genera, and eight species had significant differences ($P > 0.05$, Figure S1). Compared with TC microbiota in controls, the abundance values of Actinobacteria, Lachnoanaerobaculum, Prevotella 7, and Prevotella melaninogenica increased
in patients with GIM, whereas the relative abundance values of *Proteobacteria*, *Neisseria*, and *N. subflava* was reduced. Compared with that of the GM microbiota of controls, the relative abundance values of *Epsilonbacteraeota*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Helicobacter*, Hp 83, Hp XZ274, and *Rothiamucilaginosa DY-18* increased in patients with GIM, but the relative abundance values of *Bacteroidetes*, *Bradyrhizobium*, *Labrys*, and *Bradyrhizobium* sp. *BTAi1* decreased. In addition, compared with that in controls, the frequency of TC *Megasphaera* in patients with GIM was higher, but the frequencies of *Moraxella* and *Streptococcus salivarius* subsp. *salivarius* were lower. The frequency of GM Hp in patients with GIM was higher than that in controls.

**LEfSe** has a strong recognition function through biological statistical differences, it performs additional tests to assess and expect the biological biomarkers. First, the nonparametric factorial Kruskal Wallis sum rank test is use to detect the characteristics of significant abundance differences, and find the group-related biological biomarkers with significant differences with abundance. The LEfSe analysis was conducted to screen potential bacteria for the diagnostics of GIM (Fig. 2). The TC *Prevotella melaninogenica* was enriched in patients with GIM (Fig. 2a), and three GM *Helicobacter* species (Hp, Hp XZ274, and Hp 83) were enriched in patients with GIM (Fig. 2b). Results suggested that Hp infection might be the key repertoire during the occurrence of GIM. Thus, the effect of Hp infection on the TC/GM sharing microbiota was observed in patients with GIM. Results showed that patients with GIM and negative RUT had 7–70 TC/GM sharing OTUs with an average of 28.7, whereas patients with positive RUT had 4–76 TC/GM sharing OTUs with an average of 28.7. The number of TC/GM sharing OTUs was divided into four categories: ≤ 20, 21–40, 41–60, and ≥ 60. The distribution of sharing OTUs was significantly different between patients with positive and negative RUT ($\chi^2 = 39.992$, $P < 0.001$). The percentage of TC/GM sharing OTUs > 20 in patients with positive RUT were significantly lower than those with negative RUT (Fig. 2c, Supplementary data for Fig. 2c), suggesting that Hp infection inhibited the oral microbiota to colonize in the stomach. Two GM *Helicobacter* species (Hp XZ274 and Hp 83) were observed in the present population. Interestingly, further analysis showed that the distribution of the top 15 TC/GM sharing bacterial genera did not change significantly, but Hp-infection significantly inhibited the colonization of the relative abundance values of the three genera (*Veillonella*, *Pseudonocardia*, and *Mesorhizobium*) in the stomach (Fig. 2d, Supplementary data for Fig. 2d), and similar results were observed at several species, including *V. atypica* and *Pseudonocardia* sp. *AL041005-10* (Fig. 2e, Supplementary data for Fig. 2e).

**Significant Correlation Of Tc/gm Sharing Bacteria And Gim**

Redundancy analysis is actually constrained PCA, and a sort method developed based on correspondence analysis which combines correspondence analysis and multiple regression analysis. Thus, here used redundancy analysis to explore the effect factors of GIM development (Fig. 3). Since the present study exerted the utmost effort to matched the lifestyle factors (smoking, drinking alcohol, drinking tea and eating pickled food) to eliminate the potential influence on the microbiome, the results showed that only the TC/GM sharing bacteria had the most significant contribution to the occurrence of
GIM ($P<0.001$), and even the distinct age and BMI did not significantly contribute to the occurrence of GIM ($P>0.05$) (Fig. 3a, Supplementary data for Fig. 3a). Further analysis of the contribution of sharing bacteria to the risk of GIM showed that 15 core bacterial species were significantly associated with GIM occurrence ($P<0.05$, Fig. 3b, Supplementary data for Fig. 3b), and the 15 core bacterial species included five TC-dominant (V. atypica, S. infantis X, Haemophilus parainuenzae T3T1, Prevotella sp. oral taxon 299 str. F0039, and Prevotella melaninogenica) and four GM-dominant (Hp XZ274, Hp 83, Methylobacterium fujisawaense and Bradyrhizobium sp. BTAi7) bacteria. These data indicated that alteration of the TC/GM sharing bacteria closely link with the GIM development.

**Promotion Of Commensal Relationships Between Tc And Gm By Hp Infection**

To observe the commensal relationship between the oral cavity and stomach, in the total samples, the core bacterial species (Fig. 3b) (average abundance $>0.1\%$ and prevalence rate $>20\%$) was selected to construct the commensal network using SparCC analysis according to the previous literature [20], and fifteen bacterial species, including five bacterial species with significant differences (Bradyrhizobium sp BTAi1, Hp 83, N. subflava, Prevotella melaninogenica, and Rothiamucilaginosa DY-18), were shortlisted the correlation analysis, and the significant correlations ($P<0.05$) were presented in the commensal networks by the open source Cytoscape software (Fig. 4). Overall, 104 significant bacterial correlations were found in the controls, among which 32 correlations were in the TC microbiota (15 positive and 17 negative correlations), and 57 significantly bacterial correlations were in the GM microbiota (40 positive and 17 negative correlations). Fifteen significantly correlations (eleven positive and four negative correlations) were observed across TC and GM. Notably, seven bacteria, consisting of two positive gastric species (Methylobacterium fujisawaense and Bradyrhizobium sp. BTAi1), four negative gastric species (Halomonas pantelleriensis, Rothia mucilaginosa DY-18, Neisseria elongata subsp. glycolytica ATCC 29315, and N. subflava), and one negative TC species (Eubacterium sulci ATCC 35585), were linked with Hp 83. Further investigation showed that patients with GIM owed 89 significant correlations, including 28 TC correlations (15 positive and 13 negative correlations), 52 GM correlations (46 positive and six negative correlations), and nine significant correlations across TC and GM (seven positive and two negative correlations). For the gastric Hp 83 in patients with GIM, five significant species appeared to be negatively correlated with this toxic Hp, but gastric Methylobacterium fujisawaense and Bradyrhizobium sp. BTAi7 were positively correlated with this toxic Hp 83 in controls (Fig. 4a). These results suggested that Hp infection might affect the commensal relationship between tongue coating and stomach.

As well as known, Hp is the famous bacteria related to gastric disorders, and it can be speculated that Hp-infection have a profound impact on the bacterial symbiosis. At species level, 35 bacterial species were overall observed, here observed the effect of Hp infection on the bacterial symbiosis in patients with GIM using the SparCC correlation analysis to construct the symbiotic relationship in Hp-positive and Hp-negative patients with GIM, respectively (Fig. 4b). The commensal relationship in 23 patients with GIM and positive RUT presented 276 significant correlations, including 38 TC correlations (24 positive and 14
negative correlations), 197 GM correlations (193 positive and four negative correlations), and 41 crossed correlations (20 positive and 21 negative correlations). The commensal network in 21 patients with GIM and negative RUT presented 277 significant correlations, including 62 TC (38 positive and 26 negative correlations), 186 GM (185 positive and one negative correlation), and 29 crossed (16 positive and 13 negative correlations) correlations. Though these correlations between bacterial species did not imply the real ecological interactions, the present data indicated that Hp-infection significantly reduce the symbiotic complexity of TC microbiota but increased the correlation between TC and GM microbiota, in other word, Hp infection might play a certain role in the bacterial crosstalk between tongue coating and stomach when GIM occurred.

**Potential Biological Significance Of Dominant Microbiota**

Here suppose that the oral and gastric microbiota play a role in the development of GIM, the involved bacteria should distinctly correlate to the clinical parameters. Thus, the SparCC correlation analysis was conducted between the total 35 known bacterial species and the obtained clinical parameters to explore the potential biological significance of the microbiota (Fig. 5). In controls, GM bacteria but not TC bacteria were significantly associated with clinical features. For example, the level of serum glucose was significantly negatively related with ten gastric bacteria (e.g., *S. infantis X*, *H. parainfluenzae T3T1*, *V. atypica*, and *Prevotella sp. oral taxon 299 str. F0039*; *P* < 0.05). In patients with GIM and negative Hp infection, 11 TC and 10 GM bacteria were significantly associated with clinical features. Six TC bacteria were significantly associated with BMI (*P* < 0.05), and three GM *Helicobacter* species (Hp 83, Hp XZ274, and Hp) were all negatively related to serum PG-I/PG-II ratio (*P* < 0.05). In patients with GIM and positive RUT, six TC and five GM bacteria were significantly associated with clinical features. Among these bacteria, TC *Fusobacterium nucleatum* subsp. *nucleatum*, and *C. showae* were significantly negatively correlated with PG-I/PG-II ratio (*P* < 0.05). Moreover, TC *C. showae* was positively correlated with PG-I/PG-II ratio in patients with GIM and negative RUT but had a completely opposite direction in patients with GIM and positive RUT. These results suggested that tongue coating microbiota could reflect the pathophysiological status or possessed diagnostic value when gastric disorders, *e.g.*, GIM, occurred.

**Discussion**

Studies showed that GIM especially corpus GIM significantly increases the GC risk [21] and that GIM can independently predict the GC risk. However, many disputes about the biological mechanism of GIM remain. Giroux *et al.* believed that GIM can be regulated by transcription factors that initiate and/or maintain cellular properties, is a precursor of low-grade dysplasia, and is a result of tissue damage and environmental adaptation [22]. Meyer *et al.* persisted that the transformation of progenase-secreting cells into mucous cell metaplasia is the general repair mechanism of many epithelial cells for mucosal injury and that the continuous chronic injury, repair, and inflammation are the key events promoting GIM to develop into GC [23]. Brown *et al.* [24] proposed paligenesis in GIM development, which is a pattern of cell self-renewal and regeneration of tissues at homeostasis, with stem cells representing just one of the
diverse methods that adult tissues employ. Certainly, paligenosis is a double-edged sword: critical for repair but risky for cancer. Wang et al. [25] discussed the microbiota-induced bystander effect on the development of colorectal cancer, and more studies should be done to bridge the gaps between the underlying mechanisms of cancer initiation and commensal microorganisms. In this study, oral and gastric bacteria are speculated to be the most common cause of inflammation, injury, and repair in stomach disorders. Hp, one of the most prevalent pathogens in humans, is recognized as a class I carcinogenic pathogen leading to chronic gastritis, atrophy, gastrointestinal metaplasia, dysplasia, and GC [26]. Research revealed that gastric microbiota besides Hp are associated with the occurrence of GIM and GC [27]. Furthermore, the molecular mechanism of Hp-mediated GIM and GC has attracted extensive attention. The molecular mechanism of Hp-mediated GIM and GC remains unclear. First, elderly patients with atrophic gastritis and GIM often show a loss of Hp, but the GC risk is still higher than that in the younger population [28]. Second, even after the radical resection of Hp, how GIM causes GC independently remains unclear [29]. Finally, the radical resection of Hp cannot completely block the occurrence of GC especially in patients with precancerous lesions of GIM [30], and a latest case-control study presented an obviously altered microbiota in gastric mucosa and gastric juice during the different histological stages of Hp-negative gastric cancers [31]. These confusing phenomena suggested that other bacterial communities initiate the carcinogenesis process in patients with GIM.

This study focused on the alteration of oral and gastric microbiota in patients with GIM. Although no significant differences in microbial diversity and community structure similarity were observed in TC or GM between patients with GIM and controls, more TC/GM sharing OTUs were observed in patients with GIM than in controls. The theory of focal infection considers that oral microbiota can participate in regulating metabolic pathways via inducing inflammatory factors and linking with the occurrence of many kinds of systemic tumors [32]. For instance, Schmidt et al. found significantly increased oral Prevotella in the gut of patients with colorectal cancer and rheumatoid arthritis [33]. Our previous review summarized the increasing evidences that translocated oral microbiota play a core role in the disturbed microbial community and fuel the disorder immune response or systemic disease progression [34]. The present data suggested that GIM development is correlated with increasing migration of microbiota from tongue coating to stomach. However, further stratified analysis showed that Hp infection in patients with GIM has a lower proportion of TC/GM sharing OTUs > 20 than non-Hp infection patients, suggesting that Hp infection may inhibit the TC microbial colonization in stomach, such as Veillonella, Pseudonocardia, and V. atypica, Pseudonocardia sp. al041005-10. Epidemiological investigations in South Korea showed that negative Hp infection are closely related to advanced stage [35] and poor prognosis [36] in patients with GC. The present data suggested that Hp infection inhibits the colonization of oral microbiota and exerts some protective effects on GIM progression to GC. Thus, non-Hp microbiota may participate in the occurrence of GIM and gastric carcinogenesis.

Most studies focused on the carcinogenic virulence factors of Hp, such as CagA and VacA, but some Helicobacter species do not occupy VacA or CagA. Additionally, the detailed functions of toxin-free Helicobacter species are still unclear [37]. In this study, TC Prevotella melaninogenica and three gastric Helicobacter species (Hp, Hp 83, and Hp XZ274) are enriched in patients with GIM, and Hp XZ274 do not
have CagA. Perhaps, these *Helicobacter* species provide a good reason for the heterogeneity of gastric carcinogenesis. Thus, the genome sequence-based diagnosis of *Helicobacter* pathogens and simultaneous detection of virulence genes have high sensitivity and specificity for GC risk and prognosis [38]. Consistent with previous results [39], gastric *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* are related to GIM development, and the distribution of gastric Rhizobiales in patients with GIM significantly increases. However, the abundances of Rhizobiales and Neisseriaceae increased, whereas that of *Helicobacter* decreased. Rhizobia are a kind of soil bacteria that can induce the formation of root nodule organs of legumes and fix atmospheric nitrogen for plant growth [40], whereas the status and function of gastric rhizobia remains unclear. Considering that *Rhizobium* and *Helicobacter* are involved in the transformation of nitrogen substances, they are speculated to compete for nitrogen utilization, providing potential evidence of non-Hp bacteria involving in GIM and GC.

Oral cavity and stomach have significantly different microenvironments. *Helicobacter* species exploit their urease to decompose urea to ammonia and neutralize the gastric acid to adapt to the stomach environment. During GC development, common symptoms present esophageal reflux, bile reflux, belching, stomachache, and distention. Thus, symptoms may deeply affect the oral environment with some unclear underlying functions. *Streptococcus* and *Veillonella*, the dominant oral bacteria, play a key role in the early stages of oral biofilm formation [41] but are always observed in the gut in case of systemic malignancies with potential diagnostic value [42]. The symbiotic relationship between *S. infantis X* and *V. atypica*, which are significantly negatively correlated in TC but significantly positively correlated in GM, is altered. Further analysis showed that Hp infection evidently affects the commensal relationship in patients with GIM. For instance, gastric *C. showae* is positively correlated with *Treponema* sp. *OMZ 838* in patients with negative RUT, whereas gastric *Ottowia* sp. *oral taxon 894* is positively correlated with *Streptococcus salivarius* subsp. *salivarius* in patients with positive RUT. Hp infection significantly reduces the symbiotic intensity between *Solobacterium moorei* and *Lactobacillus reuteri I5007* in the stomach of patients with GIM. Therefore, the alteration of oral and gastric commensal relationships may reflect the stage of stomach disorders, which open a new view of microbial ecology in the precancerous lesion of gastric cancer.

During the development of precancerous lesions of GC, serum PG-I/II ratio is an important indicator for the degree of GM atrophy. Correlation analysis presented that TC bacteria are not significantly correlated with serum PG-I/II ratio, and few GM bacteria (*e.g.*, *C. showae*) are significantly negatively correlated with serum glucose in controls. By contrast, in patients with GIM and negative RUT, three GM *Helicobacter* species (Hp 83, Hp XZ274, and Hp) are significantly negatively correlated with serum PG-I/II ratio, whereas TC *C. showae* is positively correlated with PG-I/II ratio. Surprisingly, in patients with GIM and positive RUT, tongue *C. showae* is significantly negatively correlated with serum PG-I/II ratio. These results suggested that *C. showae* is involved in the occurrence of atrophic gastritis and/or GIM, and this finding is consistent with that of a previous report [11]. These results suggested that non-*Helicobacter* bacteria are involved in the occurrence of GIM, and attention should be paid on their underlying mechanism of gastric carcinogenesis.
In summary, previous studies showed that orally derived bacteria could colonize and persist in the gut, leading to chronic inflammation, and the oral health status may be a potential contributor to inflammatory diseases in the other niches including intestine and liver [42, 43], and this observational study presented the interrelationships between the oral and gastric microbiota in the GIM patients. Of course, several limitations should be considered. 1) A detailed molecular classification of Hp cannot be directly developed because of small sample capacity. 2) The limited population from a single region needs to be verified by multiregional population. 3) Bacterial analysis based on 16S rRNA gene sequencing cannot distinguish living bacteria from dead bacteria. 4) Since the high-throughput sequencing data have an arbitrary total imposed by the instrument [44], more stages of analysis should be treated as compositions, such as differential abundance analysis of microbial taxa after the transformation of these data to eliminate interference factors. Anyway, our present data provided new insights into the shared microbiota between tongue coating and stomach in the occurrence of GIM. Hp infection deeply affects the microbial crosstalk between tongue coating and stomach. Non-*Helicobacter* bacteria are also involved in the development of GIM and provides new understanding of the etiology of gastric carcinogenesis.

**Abbreviations**

<table>
<thead>
<tr>
<th>TC</th>
<th>Tongue coating</th>
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<tr>
<td>GM</td>
<td>Gastric mucosa</td>
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<td>GIM</td>
<td>Gastric intestinal metaplasia</td>
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<td>OTU</td>
<td>Operational taxonomic unit</td>
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<tr>
<td>RUT</td>
<td>Rapid urease test</td>
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<td>LEfSe</td>
<td>Linear discriminant analysis effect size</td>
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<td>GC</td>
<td>Gastric cancer</td>
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<td>Hp</td>
<td><em>Helicobacter pylori</em></td>
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<td>BMI</td>
<td>Body mass index</td>
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<td>PG</td>
<td>Pepsinogen</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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**Declarations**

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Author contributions

JZ and CC designed and supervised the study; ML, RS, ZH, and BL collected samples and clinical information; JW, ZW, ML, JX, and JZ finished sequencing and data analysis; JW, ZW, and JZ wrote the manuscript, and JZ critically reviewed and revised the manuscript. JW, ZW, and ML contributed equally to this work. ML is now working in Guangdong Province Hospital of Chinese Medicine. All authors have read and approved the manuscript.

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Ethics approval and consent to participate

All methods of this study were carried out in accordance with relevant guidelines and regulations. All of the patients enrolled in this study agreed and signed the informed consents for their clinical data and tissues to be used for scientific research, and the project was approved by the Ethics Committee of People's Hospital of Yangzhong City (No. 2018-039), and all patients had been informed and retained the signatures. Tongue coating and gastric mucosa samples were collected in the Institute of Tumour Prevention and Control, People's Hospital of Yangzhong City.

Consent for publication

Not applicable.

Availability of data and materials

The data relevant to the study are included in the article or uploaded as online supplementary information. This raw data can be found in National Center for Biotechnology Information (NCBI) BioProject (https://www.ncbi.nlm.nih.gov/bioproject/), and the accession number is PRJNA886453.

Competing interests

The authors have declared that no competing interest exists.

References


Figures
Figure 1

Altered microbiota of both tongue coating and gastric mucosa in patients with gastric intestinal metaplasia.

Microbial structures were presented at phylum (a), genus (b), and species (c) levels. The dissimilarity between tongue coating (TC) and gastric mucosa (GM) microbiota in patients with gastric intestinal metaplasia (GIM) and controls was determined by Jaccard distances ranging from 0 (completely similar) to 1 (completely dissimilar) (d). Density plots presented the distributions for the number of TC/GM sharing OTUs in controls (e) and in patients with GIM (f). The distinctly different distribution of the percentage of TC/GM sharing OTUs was observed between patients with GIM and controls (g).
LEfSe analysis of tongue coating and gastric mucosa microbiota between controls and patients with gastric intestinal metaplasia.

LEfSe analysis was conducted from phylum to species between patients with GIM and controls in tongue coating (TC) (a) and gastric mucosa (GM) (b). Based on the results of rapid urease test (RUT), positive RUT was considered as Hp (+), whereas negative RUT was considered as Hp (−). Then, the different distributions of TC/GM sharing OTUs were significantly related to the status of Hp infection ($\chi^2 = 39.992$, $P < 0.001$) (c). The top 15 sharing bacterial genera (d) and species (e) were presented in accordance with the frequencies and abundance values in Hp (+) and Hp (−) patients.

Figure 2
Figure 3

Redundancy analysis of environmental factors on development of gastric intestinal metaplasia.

Redundancy analyses were conducted to estimate the influences of environmental factors on the development of gastric intestinal metaplasia (GIM), and results showed that only the sharing bacteria between tongue coating and gastric mucosa presented significant effect on GIM development (a). Top 15 sharing bacterial species (b). The asterisk (*) indicates significant statistical value ($P < 0.05$).
Figure 4

Symbiotic relationship of tongue coating and gastric mucosa microbiota.

Symbiotic network of tongue coating (TC) and gastric mucosa (GM) in controls (a) and patients with gastric intestinal metaplasia (GIM) (b). Symbiotic relationship of TC and GM microbiota in patients with GIM and positive rapid urease test (RUT) (c) and patients with GIM and negative RUT (d). Red squares
indicate positive correlation ($r > 0$), blue squares indicate negative correlation ($r < 0$), and asterisks (*) indicate significant correlation (absolute value of $r$ is greater than 0.4, $P < 0.05$). As for the names of the network nodes, t- represents tongue coating, and g- represents gastric mucosa.

Figure 5

Analysis of potential biological significance of tongue coating and gastric mucosa bacteria.
SparCC correlation analysis was performed between 35 known bacterial species and 7 clinical features. No significant correlation was observed between tongue coating (TC) bacteria and clinical features in controls ($P > 0.05$). However, many significant correlations were observed between TC bacteria and clinical features in patients with gastric intestinal metaplasia (GIM). In 21 GIM patients with negative rapid urease test (RUT) (Hp- GIM group), 10 negative and 4 positive correlations were observed ($P < 0.05$); in 23 GIM patients with positive RUT (Hp+ GIM group), six negative and one positive correlation were observed ($P < 0.05$). Red squares indicate positive correlation ($r > 0$), blue squares indicate negative correlation ($r < 0$), and the asterisks (*) indicate significant correlation (absolute value of $r > 0.4$, $P < 0.05$). In the labeling, t- represents tongue coating, and g- represents gastric mucosa.

**Supplementary Files**

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- SupplementarydataforFigure3.xlsx
- SupplementaryTablesandFigurelegends.docx