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Bacterial overgrowth and diversification of microbiota in gastric cancer

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Objective Microbiota is potentially linked to the development of cancer. However, the features of microbiota in gastric cancer remain unclear. The aim of this study was to characterize the gastric microbiota in cancer.

Methods A total of 315 patients, including 212 patients with chronic gastritis and 103 patients with gastric cancer, were enrolled in the study. The bacterial load of gastric mucosa was determined using quantitative PCR. To analyze the biodiversity, structure, and composition of microbiota, amplicons of the 16S rRNA gene from 12 patients were pyrosequenced. The sequences were processed and subsequently analyzed.

Results The amount of bacteria in gastric mucosa was estimated to be 6.9×10^8 per gram tissue on average. It was higher in *Helicobacter pylori*-infected patients (7.80 ± 0.71) compared with those uninfected (7.59 ± 0.57, $P = 0.005$). An increased bacterial load up to 7.85 ± 0.70 was detected in gastric cancer compared with chronic gastritis ($P = 0.001$). The unweighted principal coordinate analysis showed that the structure of microbiota in gastric cancer was more diversified. Five genera of bacteria with potential cancer-promoting activities were enriched in gastric cancer. The weighted principal coordinate analysis showed that the presence of *Helicobacter pylori* markedly altered the structure of microbiota, but had little influence on the relative proportions of the other members in the microbiota.

Conclusion Findings from this study indicated an altered microbiota in gastric cancer with increased quantity of bacteria, diversified microbial communities, and enrichment of bacteria with potential cancer-promoting activities. These alterations could contribute toward the gastric carcinogenesis. Eur J Gastroenterol Hepatol 28:261–266

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Introduction

The human gut microbiota consists of a huge amount of bacteria [1]. Under physiological conditions, microbiota is vital to human health. It participates in energy metabolism, absorption of nutrients, maturation of the intestinal immune system, and protection from infection of pathogens [2,3]. Alterations in microbiota are potentially linked to cancer. Bacteria with potential cancer-inducing activities, including *Fusobacteria* and *Escherichia coli*, have been found to be increased in colorectal cancer [4,5].

The human stomach harbors a large number of bacteria in addition to *Helicobacter pylori* [6]. *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, and *Actinobacteria* are predominant in gastric microbiota, although there is considerable variation in the most abundant bacteria between individuals [7,8]. Gastric microbiota are potentially linked to the development of gastric cancer. Germ-free transgenic mice had a delayed onset of *H. pylori*-induced gastric cancer compared with specific pathogen-free mice [9]. Intervention with antimicrobial therapies delayed the onset of gastric cancer in transgenic mice irrespective of *H. pylori* infection [10]. Feeding of germ-free transgenic mice with an artificial intestinal microbiota accelerated the occurrence of cancer [9].

Gastric cancer is one of leading causes of cancer-related death. It develops through a multifactorial, multistep process [11]. *H. pylori*, a major carcinogenic pathogen of the stomach [12], initiate the mucosal inflammation, leading to mucosal atrophy and finally cancer. Using culture-dependent approaches, bacterial overgrowth in the stomach has been found with a lowered acid output [13,14]. These bacteria potentially promote the production of nitrite, leading to accumulation of carcinogenic N-nitroso compounds [15,16]. Thus, it has been supposed that overgrowth of bacteria contributes toward the development of gastric cancer [17]. However, microbiota in gastric cancer has not been well studied. The microbial diversity, structure, and composition in gastric cancer remain poorly understood. The aim of the present study was to characterize the microbial community in gastric cancer and explore its potential associations with the carcinogenesis.

Methods

Patients and sample collection

A total of 315 patients, including 212 patients with chronic gastritis and 103 patients with gastric cancer, were enrolled

European Journal of Gastroenterology & Hepatology 2016, 28:261–266

Keywords: chronic gastritis, gastric cancer, *Helicobacter pylori*, microbiota

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Received 23 July 2015 **Accepted** 2 November 2015

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in the study. Of these, 190 were men. The average age of the patients was 55.8 ± 13.5 years. All participants were selected from among those who underwent endoscopy in our hospital from March 2012 to August 2014. A written informed consent was obtained from all the participants and the study protocol was approved by the Medical Research Ethical Committee of Qingdao Municipal Hospital. To minimize the potential influence on the microbiota, all patients enrolled had not received antibiotics or proton pump inhibitors treatments 4 weeks before sample collection. For patients with chronic gastritis, those enrolled in the study had an endoscopic finding of superficial gastritis only. Those with endoscopic findings of peptic ulcer, polyps, or any other local lesions were excluded. Patients who showed histological evidence of atrophy or intestinal metaplasia were further excluded from this study. These inclusion/exclusion criteria minimized the potential compounding factors for the purpose of the study. For the enrollment of patients with gastric cancer, only those with an endoscopic finding of noncardia cancer were included. Histologically, these 103 gastric cancer cases consisted of 87 intestinal-type and 16 diffuse-type cancer. Two antral biopsies were taken from patients who underwent the endoscopy examination. For gastric cancer, biopsies were obtained from the antrum if possible or 5 cm away from the cancerous lesions. One biopsy was used for routine histological examination, whereas the other biopsy was stored at −80°C for DNA extraction. The status of *H. pylori* was determined using a modified Giemsa staining [18].

DNA extraction

To extract genomic DNA, biopsies were ground and then treated with 1 U of DNase I to eliminate any potentially foreign bacterial DNA. To increase the yield of bacterial DNA, samples were treated with lysozyme at a final concentration of 50 mg/ml. Genomic DNA was then extracted using a Qiagen DNeasy blood and tissue kit (Qiagen, Hilden, Germany).

Quantitative PCR

To determine the bacterial load in gastric mucosa, real-time quantitative PCR (qPCR) was performed to amplify the bacterial 16S rRNA gene according to the report by Harms *et al.* [19]. The following primers or probes were used in the amplification: Forward primer, 1055F (5′-ATGGCTGTCGTCAGCT-3′), the reverse primer 1329R (5′-ACGGGCGGTGTGTAC-3′), probe 16STaq1115 [5′-(6-FAM)-CAACGAGCGCAACCC-(TAMRA)-3′] [19]. The PCR reaction consisted of a total volume of 25 µl containing 1 × Premix Ex Taq (Takara, Dalian, China), 0.2 µmol/l each of primers, 0.2 µmol/l probe, and 20 ng DNA template. Cycling conditions included an initial denaturation at 95°C for 30 s, followed by 50 cycles of 95°C for 5 s and 60°C for 30 s. Standard curves were constructed with a serial dilution of a plasmid containing the full length of the 16S rRNA gene. The bacterial load was calculated as copy numbers of the 16S rRNA gene per microgram DNA. To estimate the bacterial amount in the stomach, the biopsies were weighted. The amount was calculated as the bacterial number per gram of tissue, given that the average copy number of the 16S rRNA gene in each bacterium was 3.6 [20].

To quantify *H. pylori* in the gastric mucosa, qPCR was performed essentially as described previously [21]. The sequence of primers used to amplify *ureB* was 5′-CAAAA TCGCTGGCATTGGT-3′ and 5′-CTTCACCGGCTAAGG CTTCA-3′, respectively. The probe sequence was 5′-(6-FAM)-AACAAAGACATGCAAGATGGCGTTAAAAA CA-(TAMRA)-3′. Standard curves were constructed with a serial dilution of a plasmid containing the full length of *ureB* from *H. pylori*. The amount of the bacterium was calculated as copy numbers of 16S rRNA gene per microgram of DNA.

Pyrosequencing and data analysis

To analyze the microbial communities of gastric mucosa, the variable V1-V3 region of the 16S rRNA gene was PCR amplified with primers 8F/533R, which had adapters and barcode. Amplification was carried out with 25 PCR cycles using Q5 high-fidelity DNA polymerase. Subsequently, amplicons were sequenced on a 454 GS-FLX system (Roche, Mannheim, Germany). These sequences were trimmed of sequencing primers, barcode, and adapters and filtered using the following criteria: length > 200 nt, < 9 homopolymers, < 0 ambiguous bases, and $Q_{avg} < 25$. Thus, a total of 147 001 reads were produced for these 12 samples. These reads were aligned. UCHIME was used to detect and remove chimeras. Sequences with an identity more than or equal to 97% were defined as an operational taxonomic unit. They were classified using the Ribosomal Database Project Naïve Bayes Classifier [22]. Rarefaction curves, alpha diversity, and beta diversity were analyzed using QIIME (University of Colorado, Boulder, Colorado, USA) [23]. The richness of gastric microbiota was evaluated with the Chao1 index, which reflects the theoretical number of species in a microbiota. The Shannon index, which took into account the number of species and the abundance of a species as well, was calculated to estimate the biodiversity of gastric microbiota [23]. Using Fast UniFrac analysis, both weighted and unweighted principal coordinate analysis (PCoA) were carried out to determine the similarity among the microbial communities [24]. This analysis was used to measure the phylogenetic distance between sets of taxa in a phylogenetic tree. The short read sequences are available at the website of the National Center for Biotechnology Information (study accession number: SRP060550).

Statistical analyses

SPSS and Prism (GraphPad Software Inc., La Jolla, California, USA) were used for statistical analyses and graph production. Student's *t*-test or χ^2-test was used for statistical analyses where appropriate. A *P*-value less than 0.05 was considered to be significant.

Results

Increased bacterial load in gastric cancer

The averaged log value of bacterial load in the gastric mucosa was 7.69 ± 0.64 copies per microgram of DNA. To measure the total bacterial number in gastric mucosa, all biopsies were weighted. Given that the average copy number of 16S rRNA in a bacterial cell was 3.6 [20], the

amount of bacteria in gastric mucosa was determined to be 6.9×10^8 per gram of tissue. For those 212 cases of chronic gastritis, the bacterial load in mild, moderate, and severe gastritis was 7.53 ± 0.57, 7.61 ± 0.41, and 7.69 ± 0.81, respectively. Student's *t*-test showed no significant difference ($P > 0.05$), suggesting that there was no association of the amount of bacteria with the severity of the inflammation. For gastric cancer, there was no significant difference in the bacterial load between intestinal type (7.73 ± 0.46) and diffuse type (7.87 ± 0.73) of cancer ($P > 0.05$).

Multivariable linear regression analysis showed that the presence of *H. pylori* infection had a significant impact on the bacterial load ($P < 0.05$), but age or sex had no influence (both $P > 0.05$). In these patients, the prevalence of *H. pylori* was 46.7% (147/315). The bacterial load in *H. pylori*-positive patients was 7.80 ± 0.71, which was significantly increased compared with *H. pylori*-negative patients (7.59 ± 0.57, $P = 0.005$). Moreover, the bacterial load of gastric mucosa was correlated positively with the amount of *H. pylori* ($R = 0.38$, $P < 0.001$) (Fig. 1). This suggested that the infection of *H. pylori* was a determinant of the bacterial amount of gastric microbiota.

The prevalence of *H. pylori* in chronic gastritis was 45.3% (96/212), which did not differ from that in gastric cancer (49.5%, 51/103) ($P > 0.05$). Compared with chronic gastritis, the bacterial load in gastric cancer was significantly increased ($P = 0.001$) (Fig. 2). These results showed that overgrowth of bacteria occurred in gastric cancer.

Alterations of microbiota in gastric cancer

The microbial communities of gastric mucosa from 12 patients were analyzed using high-throughput sequencing of 16S rRNA amplicons. These included six patients with chronic gastritis and six patients with gastric cancer. The Chao 1-estimated richness in gastric cancer (985.3 ± 242.6) was slightly higher than that in chronic gastritis (920.5 ± 198.7). However, the difference was not statistically significant ($P > 0.05$). Shannon's diversity index in gastric cancer (1.93 ± 0.52) was similar to that in chronic gastritis (2.07 ± 0.78) ($P > 0.05$). The structure of microbiota was explored by an unweighted unifraction analysis. The results showed that the microbiota from chronic

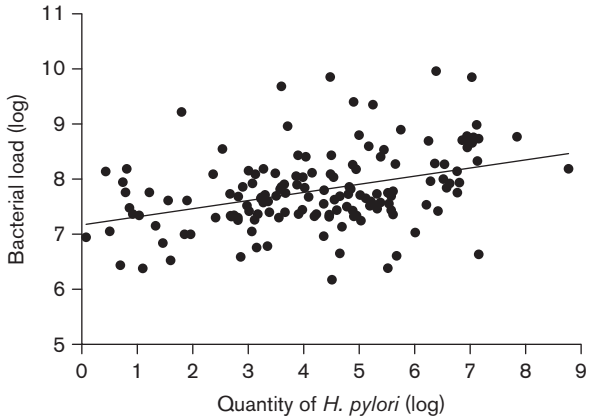

Fig. 1. Correlation of the bacterial load in gastric mucosa with *H. pylori*. Bacterial load and quantity of *H. pylori* were determined using quantitative PCR. The amount was calculated as copy numbers of the 16S rRNA gene (or *ureB* for *H. pylori*) per microgram DNA. Linear regression analysis found that the bacterial load was positively, although weakly, correlated with the quantity of *H. pylori* in gastric mucosa ($R = 0.38$, $P < 0.001$). *H. pylori*, *Helicobacter pylori*.

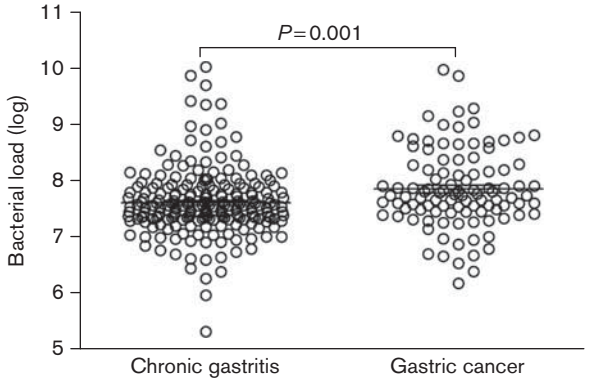

Fig. 2. The bacterial load in gastric cancer. The bacterial load of eubacteria in gastric cancer and chronic gastritis was determined using quantitative PCR. The amount was calculated as copy numbers of 16S rRNA gene per microgram of DNA.

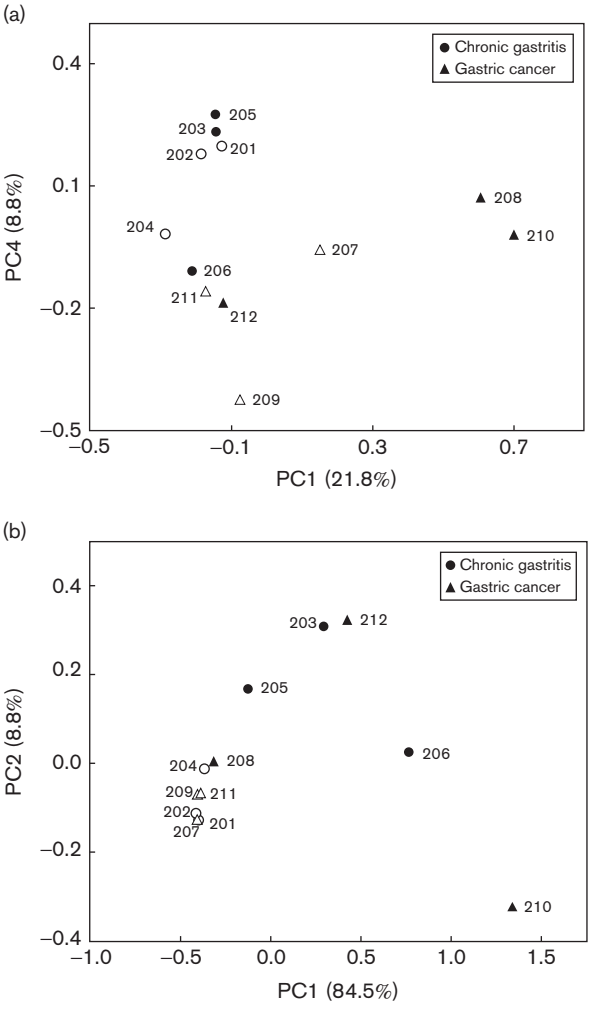

Fig. 3. The unweighted (a) and weighted (b) principal coordinate (PC) analysis of microbiota from gastric cancer and chronic gastritis using Fast UniFrac analysis. *H. pylori*-positive individuals are indicated by filled circles or triangles. *H. pylori*, *Helicobacter pylori*.

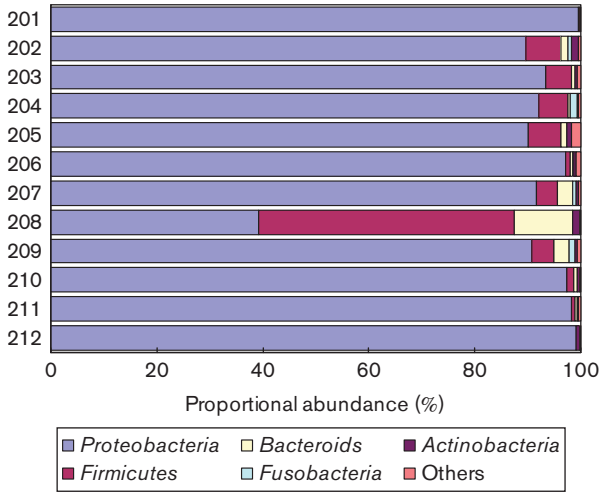

Fig. 4. Compositions of gastric microbiota at the phylum level. High-throughput sequencing of amplicons of the 16S rRNA gene was performed on 12 samples from patients with chronic gastritis (201–206) and gastric cancer (207–212).

gastritis tended to cluster together, whereas samples from gastric cancer were scattered in the plot (Fig. 3a). This suggested that the structure of microbial communities was phylogenetically diversified in gastric cancer. Compositional analyses showed that *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, and *Actinobacteria* were dominant in microbiota (Fig. 4), although the most predominant phylum varied between individuals. At the genus level, however, five genera of bacteria were enriched in gastric cancer. These included *Lactobacillus*, *Escherichia–Shigella*, *Nitrospirae*, *Burkholderia fungorum*, and *Lachnospiraceae* uncultured. Of particular interest, *Nitrospirae* was present in all patients with gastric cancer, but absent in patients with chronic gastritis.

Influence of H. pylori on microbial communities

Of these 12 patients, three with chronic gastritis and three with gastric cancer were determined to be *H. pylori* positive. In those *H. pylori*-negative patients, however, five of six had a low level of the bacterium detected (from 0.04 to 0.67%). The Chao1-estimated richness of microbiota from *H. pylori*-positive patients (999.5 ± 262.7) was not significantly different from that from *H. pylori*-negative patients (906.3 ± 163.2) ($P > 0.05$). Shannon's diversity index was markedly increased in *H. pylori*-positive patients (2.42 ± 0.58) compared with *H. pylori*-negative patients (1.56 ± 0.39) ($P < 0.05$), suggesting that the quantity of *H. pylori* could markedly influence the diversity of gastric microbiota. Weighted PCoA analysis found that *H. pylori*-negative patients tended to cluster together, whereas those infected by the bacterium were scattered (Fig. 3b). As both analyses of Shannon's diversity index and weighted PCoA analysis take into account the abundance, these results indicated that the quantity of *H. pylori* could alter the abundance of other bacteria in the microbiota. This would lead finally to an alteration of the structure of microbiota. Compositional analysis of microbiota showed no significant difference between *H. pylori*-positive and *H. pylori*-negative patients.

Discussion

In this study, we found that the bacterial load in the gastric mucosa was determined to be 6.9×10^8 per gram of tissue. It is much lower than the abundance of bacteria present in the intestine [25], indicating that the human stomach is relatively hostile to the bacterial colonization [26]. Findings from the present study, however, indicated a markedly increased bacterial load in gastric cancer. Bacterial overgrowth in the stomach has been found in various precancerous conditions [13,27], including hypochlorhydria and mucosal atrophy. It has been suggested that microbes in the stomach are involved in the production of carcinogens and promotion of inflammatory injuries [15,28] Thus, bacterial overgrowth is a potential cancer-promoting factor [17]. Nonetheless, further studies are indicated to clarify whether the bacterial overgrowth is a consequence of cancerous mucosa that generates environments favoring bacteria proliferation. Both Chao1-estimated richness and Shannon's diversity index reflect the number of species in a microbial community [29]. Our results found that they were similar between gastric cancer and chronic gastritis, indicating that there is no alteration in the number of bacterial species in the microbiota from gastric cancer. The PCoA analysis takes into account the bacteria phylogeny [29]. In contrast to chronic gastritis, our results showed a scattered pattern in gastric cancer. This indicated that members of microbiota in gastric cancer were more distantly related, suggesting a diversified microbiota harbored in gastric cancer. Taken together, these results indicate bacterial overgrowth of diversified microbiota in gastric cancer. The contribution of such alterations toward the development of cancer requires further investigations.

The results from this study found that at the phylum level, the composition of microbiota in gastric cancer did not differ significantly from chronic gastritis. Nonetheless, enrichment of five bacterial genera was found in gastric cancer. In agreement with recent studies [30,31], *Lactobacillus* and *Lachnospiraceae* uncultured were found to be more abundant in gastric cancer. This possibly reflects the reduced bactericidal capacities resulting from the lowered acid production in the stomach [32]. A number of species from *Lactobacillus* have been used as probiotics functioning in the prevention of infection by pathogens [33], alleviation of inflammation, and modulating the microbiota [34,35]. However, *Lactobacillus* is also capable of inducing inflammatory injuries of epithelial cells [36]. Thus, it requires further clarification with respect to the relationship between increased abundance of *Lactobacillus* and gastric cancer. *Burkholderia* colonizes the stomach and other organs [37]. It is reportedly associated with induction of inflammation [38,39]. Our results also found an increased abundance of *Escherichia–Shigella* in gastric cancer. A similar finding has been reported in colorectal cancer [40]. *E. coli* produces a genotoxic toxin, which promotes the development of colon cancer in mice [41]. Therefore, *E. coli* could be involved in the development of gastric cancer.

Nitrate/nitrite and their metabolites are associated with a variety of functions. Acidified nitrite is capable of killing bacteria [32]. Nitrate could shape the intestinal microbiota when acting as a source of energy [42]. Nitric oxide, a final

product of nitrite reduction, is intensively involved in the protection of mucosal integrity [43]. Importantly, N-nitroso compounds derived from metabolisms of nitrate/nitrite are potent carcinogens [15,16]. *E. coli*, *Lactobacillus*, and *Nitrospirae* are all known to play a role in the metabolisms of nitrate/nitrite [42,44,45]. As the level of nitrate/nitrite increases in the gastric cancer and its precancerous conditions [46], it could be expected that the production of N-nitroso compounds is possibly enhanced by these bacteria. Thus, these enriched bacteria could participate in the carcinogenesis.

H. pylori is a major risk factor for gastric cancer. The influence of *H. pylori* on gastric microbiota has not been fully understood. Our findings showed that *H. pylori* infection was associated with an increased amount of mucosa-associated bacteria. This is possibly caused by changes in the gastric niche induced by *H. pylori*. Otherwise, it is plausible that *H. pylori*-infected individuals have a gastric niche favoring bacterial colonization. It has been found that *H. pylori* had a major impact on the structure of gastric microbiota [31]. This is most likely caused simply by a takeover of other bacteria by *H. pylori*. When eliminating *H. pylori* from the analysis of microbiota, the abundance of other bacteria in *H. pylori*-positive patients does not alter compared with *H. pylori*-negative individuals [7,8]. In agreement with this, our study found that the diversity and structure of microbiota altered in *H. pylori*-infected stomach only when analyses took into account bacterial abundance. These results showed that the major influence of *H. pylori* on microbiota is the increased amount of bacteria in the stomach.

Gastric microbiota in cancer has been analyzed in two recent studies [30,31]. Results from this study have confirmed the findings from those studies that some bacteria were enriched in gastric cancer. Nonetheless, our study quantified the bacterial amount in gastric cancer and found bacterial overgrowth in cancer. In this study as well as in two other recent studies [30,31], microbiota has been characterized only in a small number of cases. To elucidate the nature of microbiota in gastric cancer, studies on a large cohort are indicated. In addition, findings from this study require further confirmation considering that only one biopsy was taken from each patient. This could underestimate the presence of focal atrophy, intestinal metaplasia, and *H. pylori* in gastric mucosa. Individual microbiota is potentially influenced by host BMI, smoking, or different strains of *H. pylori* [47,48]. It would be interesting to take these factors into account in future studies.

Conclusion

In summary, findings from this study showed that the microbiota in gastric cancer had an increased number of diverse bacteria. It appears that the altered microbiota potentially have cancer-promoting activities. The mechanisms and pathways by which these alterations are generated remain to be investigated in the future.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

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